THE ROLE OF DENDRITIC CELLS IN ULTRAVIOLET-B-INDUCED IMMUNOSUPPRESSION

Thesis submitted for the Degree of Doctor of Philosophy

by

Michael Benedict Lappin

University of Edinburgh

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Declaration

I declare that the studies presented in this Thesis are the result of my own independent investigation, with the exception of the Langerhans cell counts and the contact hypersensitivity measurements in the chronic UVB study which were carried out with the assistance of Dr. A. A. El Ghorr, and the HPLC analysis of urocanic acid in the chronic study which was carried out by Dr. J.C. Crosby.

This work has not been submitted for candidature for any other degree.

Michael Lappin

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Abstract

Exposure to ultraviolet-B light (UVB) suppresses immune responses to a variety of antigens, including contact sensitisers, in both mice and humans. A number of mediators of UVB-induced immunosuppression have been proposed including DNA damage, *cis*-urocanic acid, TNF-α and IL-10. UVB irradiation also causes the loss of Langerhans cells (LC) from the skin and the accumulation of LC-derived dendritic cells (DC) in the draining lymph nodes (DLN).

A chronic UVB exposure protocol, using broadband (270-350nm) and narrowband (311-312nm) sources, was used to examine the effect of UVB on the skin immune system. Exposure to both sources reduced the numbers of LC in the epidermis. Exposure to broadband but not narrowband UVB induced epidermal thickening in a dose dependent manner. Although damage measured by thickening resulted from broadband UVB only, both sources led to a small increase in sunburn cells, which may represent an apoptotic keratinocyte population. Both sources were equally efficient at inducing the conversion of *trans*-urocanic acid to the *cis*-isomer, but only the broad band source suppressed contact hypersensitivity (CH) responses. Thus LC loss and the isomerization of urocanic acid may not be the primary mediators of immunosuppression during chronic UVB exposure.

An acute UVB exposure protocol was used to examine the effect of UVB on DC *in vivo*. Mice were irradiated with an immunosuppressive dose of UVB, and the function and phenotype of DC accumulating in the DLN was examined. Exposure to UVB prior to sensitisation did not reduce the ability of DC to induce proliferative responses of hapten sensitized lymph node cells (LNC). The accessory function of DC in mixed lymphocyte reactions was also unaffected by prior exposure to an immunosuppressive dose of UVB. The expression of MHC class II, intercellular adhesion molecule-1 and B7-2 (CD86) on DC was also examined. UVB failed to affect the percentage of DC expressing these markers or the surface density of their expression. Thus UVB failed to affect the function or phenotype of DC draining the site of exposure. From these results there was no clear evidence to support the view that the immunosuppression following low-dose UVB exposure *in vivo* results from UVB-induced changes in DC .

The induction phase of CH is associated with proliferation and interleukin-6 (IL-6) production by LNC draining the sensitised site. Accumulating DC provide the main source of IL-6 within the sensitised lymph node. Since IL-6 is important in the early stages of antigen presentation, it is possible that its production by DC is important in the generation of effector T lymphocyte populations. Consistent with previous studies, it was

shown that LNC from UVB-resistant BALB/c mice proliferated and produced high levels of IL-6 in response to the sensitiser, oxazolone. In contrast, LNC from UVB-susceptible C3H/HeN mice produced low-levels of IL-6 following sensitisation but showed similar proliferative activity to BALB/c LNC. The lack of IL-6 activity was not attributable to a congenital variation between these strains with respect to IL-6 production as comparable levels of constitutive and inducible cutaneous expression of this cytokine were measured in BALB/c and C3H/HeN mice, following, respectively, topical exposure to vehicle alone or oxazolone. Sonicating LNC from sensitised BALB/c and C3H/HeN caused the release of similar levels of IL-6. The levels of intracellular IL-6 in C3H/HeN LNC following 24 hours in culture, failed to account for the deficit in IL-6 production. It is unlikely therefore that the lack of IL-6 production by C3H/HeN LNC represents an inability to secrete this cytokine. To clarify the role of IL-6 production in UVB-induced immunosuppression, further UVB-resistant (AKR) and UVB-susceptible (C57BL/6 and DBA/2) strains were examined, all of which showed low-levels of IL-6 production following sensitisation with oxazolone. Therefore no correlation was shown between the level of IL-6 production following sensitisation, and the susceptibility or resistance of mice to the immunosuppressive effects of UVB.

Abbreviations

ADP ..... adenine diphosphate
ATP ..... adenine triphosphate
AIDS ..... accquired immune deficiency syndrome
ALVC ..... afferent lymph veiled cell
AOO ..... 4.1 acetone:olive oil
APC ..... antigen presenting cell
AAMLR ..... allergen activated mixed lymphocyte reaction
BALT ..... bronchial-associated lymphoid tissue
C. albicans ..... *Candida albicans*
CH ..... contact hypersensitivity
CLA ..... common lymphocyte antigen
CPM ..... counts per minute
DBT ..... 1:1 acetone:dibutylphtalate
DC ..... dendritic cell
DETC ..... dendritic epidermal T cell
DH ..... delayed hypersensitivity
DLN ..... draining lymph node
DNFB ..... dinitrofluorobenzene
EC ..... epidermal cell
ELISA ..... enzyme-linked immunosorbant assay
FITC ..... fluorescein isothiocyanate
FCS ..... foetal calf serum
GAG ..... glycosaminoglycan
GALT ..... gut-associated lymphoid tissue
GM-CSF ..... granulocyte-macrophage colony-stimulating factor
HIV ..... human immunodeficiency virus
HPLC ..... high performance liquid chromatography
HSV ..... herpes simplex virus
ICAM-1, 2, 3 ..... intercellular adhesion molecule-1, 2, 3
IFN-γ ..... interferon-gamma
Ig ..... immunoglobulin
IgE ..... immunoglobulin-E
IgG ..... immunoglobulin-G
IL-1 ..... interleukin-1
IL-2 ..... interleukin-2
IL-3 ..... interleukin-3
IL-4 ..... interleukin-4
IL-5 ..... interleukin-5
IL-6 ..... interleukin-6
IL-8 ..... interleukin-8
IL-10 ..... interleukin-10
IL-12 ..... interleukin-12
KLH ..... keyhole limpet haemocyanin
LC ..... Langerhans cells
LFA-1 ..... lymphocyte function-associated antigen-1
LNC ..... lymph node cells
LPS ..... lipopolysaccharide
MAIDS ..... mouse accquired immunodeficiency syndrome
MECLR ..... mixed epidermal cell lymphocyte reactions
MED ..... minimal erythemal dose
MHC ..... major histocompatibility locus

MIP-2 ..... macrophage inhibitory protein-2
MLR ..... mixed lymphocyte reactions
NK ..... natural killer
OVA ..... ovalbumin
PBMC ..... peripheral blood mononuclear cell
PBS ..... phosphate buffered saline
RLV ..... Raucher leukemia virus
RT ..... room temperature
SALT ..... skin-associated lymphoid tissue
SCC ..... squamous cell carcinoma
SD ..... standard deviation
SEM ..... .standard error mean
S. Mansoni ..... *Shistosoma mansoni*
SIS ..... .skin immune system
SLS ..... sodium lauryl sulphate
SS ..... side scatter
TBS ..... tris buffered saline
TCR ..... T cell receptor
TNF-α ..... tumour necrosis factor-alpha
T. Spiralis ..... *Trichinella spiralis*
UCA ..... urocanic acid
UV ..... ultraviolet
VCAM-1 ..... vascular cell adhesion molecule-1
VLA-4 ..... very late activation-4
VSG ..... variable surface glycoprotein

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Chapter 1 Introduction

This chapter provides a general introduction to cutaneous immunity and in particular describes the role of dendritic cells within the skin immune system. In addition some background information on ultraviolet light (UV) is included. A detailed review of the literature relating to UVB and the suppression of skin immune responses follows, which should provide a clear understanding of the concepts which underlie the work contained in this thesis.

1.0 The skin immune system

1.0.1 Skin physiology

The skin is the largest organ in the body providing a relatively impermeable barrier to the external environment. The skin consists of the dermis, mainly connective tissue perfused by blood and lymph vessels, which is overlaid by the epidermis. The physical structure of the epidermis is provided by keratinocytes in distinct stages of differentiation. The most terminally differentiated keratinocytes form the horny layers of the skin called the stratum corneum, which together with substances on the skin surface, provides defence against many chemical and biological agents.

1.0.2 Skin associated lymphoid tissue (SALT)

The skin is an important point of entry for infectious microorganisms and is subject to the actions of environmental factors that can act as tumour initiators and promoters. Therefore the skin requires active immune function to provide protection against infection and immune surveillance of transformed cells. Like other organs in the body, notably the gut and respiratory tract, the skin has its own associated immune system.

The concept of SALT was first suggested by Streilein in 1978. The hypothesis was built on two observations, firstly that lymphoid malignancies which showed

cutaneous tropism were always T cell derived, and secondly the identification of a population of so-called 'passenger leukocytes' within the skin. These passenger leukocytes mediated graft rejection when skin from chimeric mice was grafted onto non-chimeric recipients. Streilein, knowing that specific T cell migration patterns existed for both gut and bronchial associated lymphoid tissue (GALT and BALT), hypothesised the existence of a third T-cell recirculation pathway, serving the skin, under the control of SALT (Streilein, 1978). This hypothesis was later confirmed with the discovery of T cell populations which expressed the marker cutaneous lymphocyte antigen (CLA) a ligand for E-selectin, which mediated their migration into the skin (Rossiter *et al.*1994). The identification of CLA and its ligand meant that dermal high endothelial venules which express E-selectin, and which mediate T cell infiltration into the skin, were included in SALT.

As more came to be known about the skin, key-components of the skin immune system were revealed. In 1980 came the first direct evidence that the epidermal Langerhans cell (LC), which was first identified over 100 years previously (Langerhans, 1868), was required for skin immune function (Toews *et al.*1980). This work and later studies, showed that the migration of antigen-bearing LC to the skin-draining lymph nodes was vital for the induction of skin immune responses. Now, the LC has been joined by other cells such as dermal dendritic cells and macrophages which have been proposed as alternative cutaneous APC populations (Streilein, 1991)

By the late eighties and early nineties there was increasing evidence that keratinocytes were an important source of cutaneous cytokine production and there were suggestions that these cytokines might be important in the control of cutaneous immune responses (Streilein, 1991). This has proved to be the case and their role in the SIS will be discussed more fully later in the introduction.

Therefore these features; specialised skin-homing T cells, LC and other cutaneous APC, skin-draining lymph nodes and keratinocytes are the component features of SALT and are required for the induction of skin immunity (Figure 1.1).

1.0.3 Cutaneous immune responses

Contact hypersensitivity (CH) responses are used commonly as a model to explore the integrity of cutaneous immune responses. The induction of CH follows the application of a sensitising chemical (contact allergen) to the skin. Chemicals, because of their simple structure and small size are generally not recognised by the immune system in their native form. However, the individual molecules (haptens) of sensitising chemicals are reactive, forming bonds with proteins in the skin to produce hapten derivatised self-proteins. These derivatised proteins act as epitopes, generating a hapten/protein-specific immune response. The induction of a CH response following a primary exposure to a hapten requires the presentation of the antigen in the DLN. Hapten derivatised proteins are processed in the skin by APC including LC (section 1.1.2.2), the LC then receive a signal to migrate out of the skin (section 1.3.2) and antigen-bearing LC migrate out of the skin and present the antigen to T cells in the DLN (sections 1.4, 1.5).

The antigen-processing pathway for CH responses is thought to model the mechanisms of antigen presentation for other antigens. DH responses are induced following the intradermal injection of an immunogenic antigen (usually protein). Foreign protein, like hapten derivatised protein, is processed by APC in the skin, these APC then migrate out of the skin and activate effector T cells in the DLN.

In the DLN, LC cause the proliferation and differentiation of T cells into an effector population with specificity for the skin (section 1.5.3). These T cells provide an immunological memory for the antigen, and, following a secondary sensitisation with the same hapten , they will be activated by interactions with hapten-bearing APC in the periphery. These activated T cells then help orchestrate the effector response.

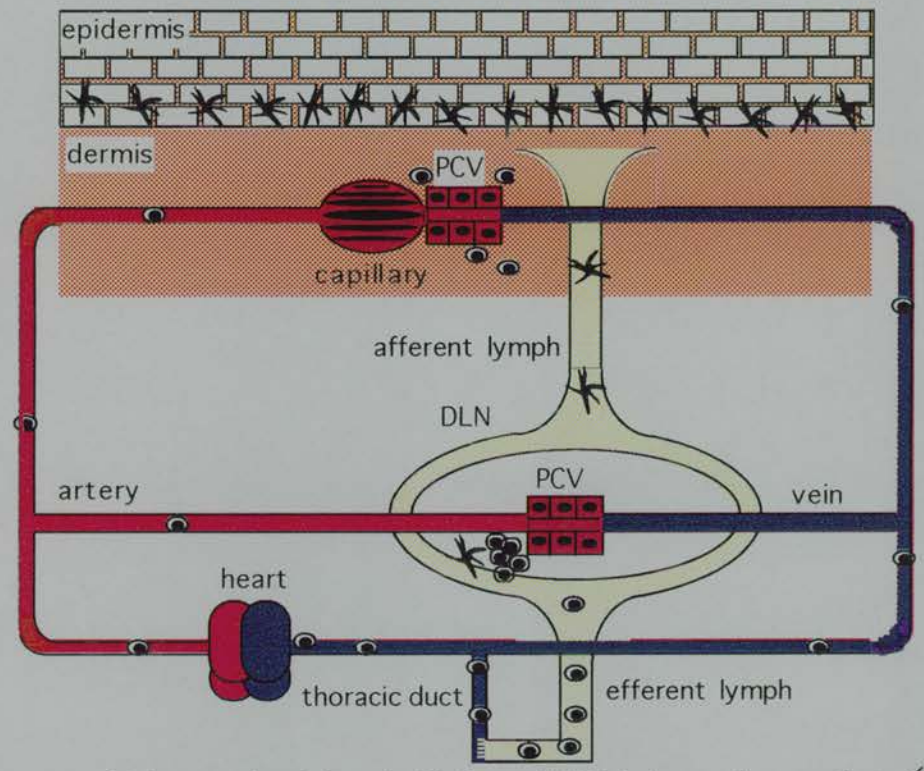

Figure 1.1 Constituents of skin associated lymphoid tissue (SALT). Langerhans cells (✳) and keratinocytes (▭) in the epidermis. Other cells such as dendritic epidermal T cells, dermal dendrocytes, macrophages and dermal 'LC-like' cells may also have a role in SALT. Skin draining lymph nodes (DLN) containing naive antigen responsive T cell clones (◉). Circulating effector T cells, with certain T cells recirculating via the skin through post-capilary venules (PCV) in the dermis. The figure is adapted from Streilein, 1991.

In most experimental protocols the effector phase of CH is measured 5-7 days following the original hapten sensitisation, when the animal is challenged with a sub-inflammatory concentration of the same chemical. The site of challenge is usually the ears or the foot pad, as it is easy to quantitate the inflammatory reaction by measuring the increase in ear or footpad thickness. The effector phase of DH responses are measured later, between 8-14 days, again by measuring increases in ear or footpad thickness following challenge. In humans CH responses are generally measured by scoring the features of inflammation (erythema, oedema, vesicle formation, epidermal necrosis) following clinical examination (Tie *et al.*1995).

CH and DH responses are cell-mediated and can be transferred to naive syngeneic animals with T cells populations from the spleen and lymph nodes of sensitised animals. The effector phase of both CH and DH responses are associated with the infiltration of T cells and cells of the monocyte/macrophage lineage into the dermis and epidermis. The activation of macrophages by T-cell-derived IFN-γ causes them to produce pro-inflammatory cytokines and increases their phagocytic activity. This infiltration of T cells and activated macrophages into the skin is the main effector response, and results in the clinical features associated with CH and DH responses.

1.0.3.1 Effector cells of CH and DH responses

The identification of the effector cell population which induces CH responses is complicated by conflicting data. The classical CH effector cell which induces responses 24-48hrs after challenge is a $CD3^+$, $CD4^+$, TCR $\alpha\beta^+$ and IL-$2R^+$ T cell (Ptak *et al.*1996). The role of CD4 T lymphocytes has been confirmed using CD4 knockout mice which displayed reduced CH responses to DNFB (Cavanagh *et al.*1996). Sensitisation in these mice was associated with increased numbers of $CD8^+$ and $CD4^-$ $CD8^-$ double negative cells. However, work by another group suggests a role for $CD8^+$ T cells in CH responses. Monoclonal antibodies recognising CD4 and CD8 T lymphocytes were administered to BALB/c mice to deplete the relevant cell population

(Gocinsky and Tigelaar, 1990; Xu *et al.* 1996). Mice depleted of CD4 T cells showed more intense and longer lasting CH responses than control animals, indicating that CD4 T cells could act as negative regulators of CH responses. Importantly, the depletion of CD8 T cells abrogated CH responses, suggesting that these cells are required for effective responses. The second study concluded that the induction of CH responses was accompanied by the generation of IFN-γ producing CD8 effector cells and IL-4 and IL-10 producing regulatory $CD4^+$ T cells (Xu *et al.* 1996).

Since CD8 cells are considered to be MHC class I restricted, this result challenges the theory that MHC class I is involved only in the presentation of intracellular contents to the immune system. Though MHC class I undoubtedly fulfils this role, the possibility that CD8 cells can act as effector cells in responses to an exogenous antigen suggest a wider role for immunity mediated via class I MHC presentation. Reactive chemicals gain immunogenicity by binding with self proteins in the skin. It remains possible that the generation of $CD8^+$ effector cells by these sensitising chemicals is the result of mechanisms not available to other groups of antigens. For instance chemical haptens may bind the self proteins which are present in the binding groove of MHC class I on the surface of cells. The derivatisation of self proteins in these grooves, particularly on migrating APC populations, may result in CD8 responses without the requirement for the antigen to be present in the cytoplasm. Alternatively, un-derivatised haptens may be able to penetrate cellular membranes and derivatise proteins in the intracellular compartment.

The effector phase of DH responses is similar to that of CH responses and the ability to transfer DH responses with T cells, suggests that these cells are the primary mediators. Since IFN-γ production is vital for CH and DH responses it is likely that similar effector T cell populations are induced in both responses. Classically DH responses are mediated by $CD4^+$ T-lymphocytes, however it is possibly that IFN-γ secreting $CD8^+$ cells may also play a role.

1.1. Dendritic cells.

1.1.1 The dendritic cell lineage

DC which were named because of their dendritic morphology, form APC populations present at low concentrations (1-2%) in the blood (blood DC), afferent lymph (veiled cells), lymphoid organs such as the spleen, lymph node, tonsil and thymus (interdigitating cells) and non-lymphoid organs such as the heart, lung, liver, gut and dermis (DC) and the epidermis (LC) (Steinman, 1991). The enrichment of DC relies on their low buoyant density, lack of adherence to plastic or the absence of markers found on other leukocytes (Steinman, 1991). These techniques have been used alone or in combination to produce enriched and highly enriched DC populations.

There are few reliable markers for DC. Positive identification of DC requires examination of the morphology, function and phenotype of cells. MHC class II is used as a marker of LC in normal skin (Klareskog *et al.*1977). LC have also been identified by their ATPase activity (Chaker *et al.* 1984), Birbeck granules (Birbeck *et al.* 1961) and in humans by their expression of CD1a (Furue *et al.* 1992). All DC populations share a number of distinguishing features. DC exhibit specialised APC activity with the ability to activate naive T cells, though some DC gain this ability following a functional maturation stage. This specialised accessory activity means that DC are very potent APC for a variety of *in vitro* proliferative responses. Associated with this activity, DC express very high levels of MHC gene products including MHC class I and II and CD1 (Steinman, 1991; Witmer-Pack *et al.* 1988; Cumberbatch *et al.* 1991), along with high levels of accessory molecules such as (intracellular adhesion molecule-1) ICAM-1, lymphocyte function associated (LFA) antigens and members of the B7 family of molecules. In addition DC lack markers for T cells (CD3), B cells (CD19), NK cells (CD16) and macrophages (CD14) (Steinman, 1991).

DC are bone marrow derived (Katz *et al.*1979) and arise, in common with macrophages and granulocytes, from a bone-marrow myeloid progenitor (Inaba *et al.*1993). Granulocyte/macrophage-colony stimulating factor (GM-CSF) alone or in

combination with interleukin (IL)-1, tumour-necrosis factor-α (TNF-α) and IL-4 seems to be important for the generation of DC progenitors *in vitro* (Inaba *et al.* 1993) and may be important in the differentiation into DC *in vivo*.

The prime function of all non-lymphoid DC is to inform the immune system of events happening in the periphery. Injury and antigen exposure causes antigen-bearing DC to migrate out of organs via the afferent lymph or blood to lymphiod organs. In the lymphoid organs DC associate with T cells and initiate immune responses. Because DC are specialised APC they can associate with, and activate naive T cells , a unique ability which means that DC are vital for the induction of primary immune responses.

1.1.2 Langerhans cells

LC are regularly spaced throughout the epidermis forming a semi-continuous network via long dendritic processes. LC are defined by their dendritic morphology and the presence of a unique intracytoplasmic organelle, the Birbeck granule (Birbeck *et al.*1961). In addition to their appearance, they are distinguished by their phenotype being MHC class II$^+$, CD45$^+$, Thy-1$^-$, CD3$^-$ (Elbe *et al.*1989), CD11b$^+$, with Fc receptors for IgG (Stingl *et al.*1977) and IgE (Bergstresser *et al.*1995) (Table 1.1). Human LC express CD1a and stain faintly with anti-CD1c (Furue *et al.*1992).

1.1.2.1 Evidence suggesting that Langerhans cells play an important role in cutaneous immunity.

The first clues that LC were involved in cutaneous immunity came from experiments examining contact sensitivity responses in mice. When haptens were applied through sites naturally deficient in LC expression, such as the hamster cheek pouch epithelium and mouse tail skin, specific unresponsiveness to hapten challenge was observed (Toews *et al.*1980; Bergstresser *et al.*1980; Streilein and Bergstresser, 1981). It was noted, that a regime of UVB treatment could deplete LC from normal skin sites (Toews *et al.*1980). Four consecutive doses of UVB radiation

Surface marker	murine LC	human LC	DETC	dermal dendrocyte	Dermal LC-like
MHC class I	√ Crowley *et al.* 1989				√ Duraiswamy *et al.* 1994
MHC class II	√ Rowden, 1980; Crowley *et al.* 1989	√ Rowden *et al.* 1977	× Bergstresser *et al.* 1993	√ Braverman and Keh-Yen, 1992	√ Duraiswamy *et al.* 1994
CD45	√ Takashima *et al.* 1995	√ Wood *et al.* 1991	√ Tschlachler *et al.* 1989		√ Duraiswamy *et al.* 1994
CD3	× Schuler and Steinman, 1985		√ Bergstresser *et al.* 1983		× Duraiswamy *et al.* 1994
Thy-1	× Schuler and Steinman, 1985		√ Tschlachler *et al.* 1983; Bergstresser *et al.* 1983		
γδ TCR	× Takashima *et al.* 1995		√ Kuziel *et al.* 1987		
ICAM 1	√ Cumberbatch et al 1992	√ De Panfilis *et al.* 1990		× induced bu IFN-γ. Cerio *et al.* 1989	
ICAM 2		× Hart and Pricket, 1993			

Table 1.1 (i). Phenotype of cutaneous dendritc cell populations (√ expression, × no expression)

Surface marker	murine LC	human LC	DETC	dermal dendrocyte	Dermal LC-like
ICAM-3		√ Teunisen *et al.* 1995			
LFA-1	√ $CD11a^-/CD18^+$ Witmer-Pack *et al.* 1988				
MAC-1	√ Schuler and Steinman, 1985	√ De Panfilis, 1989		× Cerio, 1989	$CD11b^-$ Duraiswamy *et al.* 1994
P150/95	√ Agger *et al.* 1990	√ De Panfilis *et al.* 1989			√ Duraiswamy *et al.* 1994
LFA-3		√ weak De Panfilis *et al.* 1991			
α1		√ α1β1 weak on 40% of LC. Staquet *et al.* 1992; × Zambruno *et al.* 1991			

Table 1.1 (ii). Phenotype of cutaneous dendritc cell populations (√ expression, × no expression)

Surface marker	murine LC	human LC	DETC	dermal dendrocyte	Dermal LC-like
α2		√ α2β1 weak on 40% of LC. Staquet *et al.* 1992; × Zambruno *et al.* 1991			
α3		√ α3β1 weak on 40% of LC. Staquet *et al.* 1992; × Zambruno *et al.* 1991			
α4	√ Aiba *et al.* 1990	√ weak. De Panfilis *et al.*1991; √ α4β1. Staquet *et al.* 1992			
α5		√ α5β1. Staquet *et al.* 1992; × α5β1. Caux *et al.* 1994a			
α6		√ weak. De Panfilis *et al.*1991; √ α6β1. Staquet *et al.* 1992			

Table 1.1 (iii). Phenotype of cutaneous dendritc cell populations (√ expression, × no expression)

Surface marker	murine LC	human LC	DETC	dermal dendrocyte	Dermal LC-like
β1		√ Zambruno *et al.* 1991			
CD1a		√ Furue *et al.* 1992		√ Cerio, 1989	√ Cooper *et al.* 1995
CD1b		√ Furue *et al.* 1992			
CD1c		√ Furue *et al.* 1992			√ Cooper *et al.* 1995
Heat stable antigen	√ Enk and Katz, 1994				
E-cadherin	√ Tang *et al.* 1993	√ Blauvelt *et al.* 1995			
FcγRII	√ Takashima *et al.* 1995				√ Cooper *et al.* 1995
FcγRIII	√ Takashima *et al.* 1995				

Table 1.1 (iv). Phenotype of cutaneous dendritc cell populations (√ expression, × no expression)

Surface marker	murine LC	human LC	DETC	dermal dendrocyte	Dermal LC-like
FcεRI		√ Bieber *et al.* 1992			
FcεRII	Takashima *et al.* 1995	√ Bieber *et al.* 1992			
CD80 (B7-1)	× Inaba *et al.* 1994; Larsen *et al.* 1992	× Symington *et al.* 93			
CD86 (B7-2)	√ (weak) Inaba *et al.* 1994; × Caux et al. 1994a				
Factor XIII				√ Braverman and Keh-Yen, 1992	

Table 1.1 (V). Phenotype of cutaneous dendritc cell populations (√ expression, × no expression)

(100 J/m^2/day) caused a highly significant depletion of ATPase$^+$ epidermal LC in mice (Toews *et al.*1980). Haptens applied to UVB treated skin induced hapten specific unresponsiveness on subsequent challenge to an unirradiated site (Toews *et al.*1980). The ability of UVB to induce unresponsiveness was dependent on the strain of mouse used, leading to the classification of UVB 'susceptible' and 'resistant' strains (section 1.7.5).

1.1.2.2 Antigen processing in the epidermis by LC.

The first stage in the recognition of antigen by the immune system is the processing of that antigen by APC. Processing refers to the internalisation and degradation of antigen into immunogenic peptide fragments which are then displayed with MHC molecules. This takes place intracellularly in acidified endosomes / lysosomes, although the exact compartment where peptides and MHC class II associate has not yet been defined. Freshly isolated murine LC are able to process and present the protein ovalbumin (OVA), to an OVA peptide specific MHC-restricted T cell hybridoma *in vitro* , an ability that is lost during culture (Streilein and Grammer, 1989). LC taken from the epidermis and cultured for 72 hrs are much less efficient at processing native OVA (Streilein and Grammer, 1989). Similarly, the ability to process antigen from *Leishmania major* amastigotes is found in freshly isolated murine epidermal LC, but is lost after 12 hrs in culture (Moll, 1993). Haptens such as nickel are able to bind MHC class II associated peptides directly (Sinigaglia, 1994) suggesting that antigen processing may not be required for all forms of contact sensitisation. As LC have phagocytic activity when freshly isolated, it seems likely that epidermal LC have the capacity to process native antigen *in vivo*. In contrast, like cultured LC, murine lymph node DC are generally poorly phagocytic, although freshly isolated splenic DC show phagocytic activity for zymosan and latex beads (Reis e Sousa *et al.*1993).

1.1.3 Dendritic epidermal T cells (DETC)

DETC are found in the epidermis of rodents where they comprise 1-2% of the cells (Bergstresser *et al.*1983; Tschachler *et al.*1989; Shimada *et al.*1992), and are CD45$^+$, Thy-1$^+$ and CD3$^+$. The CD3 molecule is predominantly associated with the 35KD/45KD TCR γ/δ heterodimer (Tschachler *et al.*1989). DETC do not express CD4 or CD8 antigens (Tschachler *et al.*1989), nor MHC class II (Elbe *et al.*1989). Their T cell lineage is confirmed by evidence that numbers of DETC are reduced significantly in athymic nude mice and that they proliferate *in vitro* when stimulated with a mixture of concanavalin A and interleukin-2 (IL-2) (Tschachler *et al.*1989). It has been suggested that DETC are involved in the induction of tolerance (Welsh and Kripke, 1990) and that the ratio of DETC to LC in the epidermis influences the intensity, but not the duration, of skin sensitisation in mice (Bigby *et al.*1987).

1.1.4 Dermal dendritic cells (DDC)

There are a number of cell types including macrophages, fibroblasts, dermal APC, and stromal cells that exhibit dendritic morphology in the dermis. In the past these populations may have been grouped together under the convenient, but uninformative, classification of dermal dendrocytes. In human skin they are factor XIII antigen$^+$, sometimes express class II antigen (Braverman and Keh-Yen, 1992) and are CD1a$^+$, CD1b$^+$ and CD1c$^+$ (Bergstresser *et al.*1995). Factor XIII is involved in scab formation, crosslinking fibrin with structural proteins, which suggests a possible role for dermal dendrocytes in wound healing. Although the term dermal dendrocyte describes more than one cell type, a human LC-like dermal APC has been found that shares dendrocyte features. This population makes up around 2.7% of total dermal cells and expresses LC-associated markers (MHC class II$^+$, CD45$^+$, CD1a$^+$, CD1c$^+$) as well as markers not expressed by LC (CD1b$^+$, CD11b$^+$ and factor X111a$^+$) (Meunier *et al.*1993). In another human study three distinct DDC populations were found within the factor XIIIa$^+$ subset. The majority of such cells (65-75% XIIIa$^+$ cells)

did not express the myelo-monocytic marker CD14 or LC marker CD1a. The second subset that made up around 15-20% of these cells expressed CD1a but not CD14 and may correspond to the LC-like subset already described. The final subset expressed CD14 but not CD1a, suggesting a monocyte/macrophage lineage (Nestle *et al.*1993).

Similar populations are found in mice, with 2.5% of cells in the normal murine dermis being MHC class II^+ (Duraiswamy *et al.*1994). The MHC class II^+ population comprises two phenotypically distinct lineages. Both populations are MHC class II^+, $p150/95^+$ (used as a DC marker), $CD45^+$ (bone-marrow derived) but $GR\text{-}1^-$ (non-neutrophil) and $CD3^-$ (Duraiswamy *et al.*1994). However, one subset expresses CD11b and the other does not. The $CD11b^+$ subset expresses Ly6c a monocyte/endothelial antigen, has no activity in syngeneic mixed lymphocyte reactions (MLR) and is thought to be a dermal monocyte derived population. Cells in the $CD11b^-$ subset act as potent stimulators in the syngeneic MLR and have dendritic morphology, but do not display Birbeck granules (Duraiswamy *et al.*1994). Unlike the mouse, CD11b is expressed by both macrophages and LC-like cells in the human dermis (Meunier *et al.*1993) and so can not be used to differentiate dermal macrophages from dermal LC-like APC. However, the expression of CD1c (a marker expressed by epidermal LC) on dermal MHC class II^+ cells marked a population which did not co-express CD36 and which were the most potent accessory cells for alloantigen responses in the dermis (Meunier *et al.*1993).

Therefore, in both murine and human dermis there exist a number of dendritic cell populations, including heterogeneous MHC class II populations which can be differentiated phenotypically by their accessory function. The MHC class II^+, $CD11b^-$ and MHC class II^+, CD1c LC-like dermal cell populations in mice and humans respectively still require further classification. It is uncertain whether these dermal APC represent a separate APC, population or if they are a migratory DC population trafficking to, or from, the epidermis. However, there is increasing evidence that the

dermis may be important in the regulation of cutaneous immune responses, a hypothesis which will be addressed again later in the introduction with reference to UVB.

1.2. Other cutaneous APC

1.2.1 MHC Class II$^+$ keratinocytes

Prior to the work showing that freshly isolated LC were capable of processing native antigen (section 1.1.2.2), the lack of phagocytic activity in lymphoid DC led to speculation about the identity of the cell population that processed antigen in the epidermis with the suggestion that keratinocytes may provide this function (Luger *et al.*1983; Streilein, 1991). Since freshly isolated LC are capable of phagocytosis, it seems unlikely that epidermal LC would require a source of peptides. However, processing by keratinocytes may affect the immune response, depending on whether the peptide fragments are degraded, released into the extracellular matrix or presented on the surface of keratinocytes. Although keratinocytes do not express MHC class II molecules constitutively, they can be induced to do so by interferon γ (IFN-γ) (Nickoloff and Turka, 1994). MHC class II bearing keratinocytes can provide accessory function for T cells that have been stimulated previously with superantigens or anti-CD3 monoclonal antibody (Nickoloff and Turka, 1994). Keratinocytes are therefore equipped to play an important role in secondary, but not primary, immune responses.

1.2.2 Macrophages

Monocyte and macrophage populations are found in the dermis of mice and humans. In the human dermis there are MHC class II$^+$, CD45$^+$ CD11b$^+$, CD36$^+$ macrophages which are distinguished from the LC-like APC (section 1.1.4) by their lack of CD1c expression (Meunier *et al.*1993), or by their expression of CD14 but not CD1a (Nestle *et al.*1993). There are two monocyte/macrophage populations in the

normal murine dermis one of which has been described in section 1.1.4 as an MHC class II$^+$, GR-1$^-$, CD11b$^+$ cell population which makes up 1.7% of the total dermal cell population (Duraiswamy *et al.*1994). The other macrophage population shows similar expression of CD11b and lack of GR-1 but in addition fails to express MHC class II (Duraiswamy *et al.*1994). Although these macrophage populations generally lack accessory cell activity (Duraiswamy *et al.*1994; Nestle *et al.*1993; Meunier *et al.*1993), they may be important in the regulation of cutaneous immune responses. Indeed, the expansion of these populations in the dermis following UVB irradiation may be relevant to UVB-induced immunosuppression and will be covered in section 1.8.1.2.

1.3. Cutaneous microenvironment

1.3.1 Factors that support LC presence in epidermis

It is interesting that while LC constitute a long-lived population in the skin, donor LC surviving for months in skin allo-grafts (Kreuger and Emam, 1984) their lifespan *in vitro* is short with their viability dropping to less than 30% following 3 days of culture (Schuler and Steinman, 1985; Heufler *et al.*1988). This has provoked interest in the mechanisms by which LC are maintained in the epidermis, particularly in the role of keratinocyte-derived cytokines in the promotion of LC viability. GM-CSF, a cytokine produced constitutively by keratinocytes (Matsue *et al.*1992) plays an important role in maintaining the viability of LC *in vitro*. Enriched murine LC cultured in the presence of recombinant GM-CSF or keratinocyte conditioned medium (KCM) showed around 60% viability on day 3 of culture compared with around 10% viability in cultures lacking GM-CSF (Heufler *et al.*1988). In the same study the addition of a neutralising antibody to GM-CSF to cultures together with the KCM abrogated the increased viability.

TNF–α is another cytokine produced constitutively by keratinocytes (Enk and Katz, 1992c; Matsue *et al.*1992) which may be important in maintaining epidermal LC

viability. Murine TNF-α increased the survival rate of murine LC *in vitro* in a GM-CSF independent manner, but unlike LC cultured with GM-CSF or in bulk epidermal cell (EC) culture, did not show the increased accessory activity in a MLR (Koch *et al.*1990). However, there is conflicting evidence from an *in vitro* model in which human peripheral blood mononuclear cells (PBMC) were cultured in GM-CSF and IL-4 to produce DC lines. These DC lines showed a number of similarities to immature DC such as LC, particularly in their ability to capture and process antigen (Steinman *et al.*1995). Exposure of these DC to TNF-α increased their accessory activity in MLR but reduced their ability to present soluble antigens (Steinman *et al.*1995), which is likely to be due to the ability of TNF-α to down-regulate antigen capture mechanisms such as macropinocytosis (Sallusto and Lanzavecchia, 1994; Caux *et al.*1994a).

1.3.2 Signals that cause the migration of Langerhans cells out of the skin.

A number of stimuli, including UVB and contact sensitisers, causes the induction or up-regulation of cytokines including IL-1α, IL-3, IL-6, IL-8, GM-CSF, IFN-α, TNF-α and TGF-β by keratinocytes (Bos and Kapsenberg, 1993; Enk and Katz, 1992c; Köck *et al.*1990). Using polymerase chain reaction (PCR), it was shown that topical exposure of mice to contact sensitisers resulted in increased epidermal mRNA for IL-1α, IL-1β, GM-CSF, TNF-α, macrophage inflammatory protein-2 (MIP-2), interferon-induced protein-10 (IP-10) and I-Aα (Enk and Katz, 1992b; Enk and Katz, 1992c). Tolerogens and chemical irritants also induce an increase in epidermal mRNA for some, but not all, of these cytokines (Enk and Katz, 1992b).

Interestingly a LC derived cytokine seems to be an important initiating signal for this cascade of epidermal cytokines. The expression of IL-1β mRNA in the epidermis is up-regulated within 15 minutes of hapten application, preceding changes in TNF-α mRNA expression which are found after 30 minutes (Enk and Katz,

1992b). Depletion of epidermal cell subsets revealed that IL-1β activity is mainly restricted to LC (Enk and Katz, 1992c). When IL-1β is injected intradermally, it causes similar changes in cytokine production to those that occur after sensitisation, with increased expression of mRNA for IL-1α, MIP-2, IL-10, TNF-α and Class II, while intradermal injection of IL-1α or TNF-α did not affect the cytokine pattern (Enk *et al.*1993). In addition, a neutralising antibody to IL-1β was able to block sensitisation (Enk *et al.*1993), suggesting that LC-derived IL-1β is an important initiation signal for the induction of contact sensitisation.

The migration of LC out of the epidermis seems likely to be TNF-α dependent, though there is still controversy surrounding this issue. As stated previously message for TNF-α is up-regulated following exposure to contact sensitisers, UVB and possibly physical injury. An intradermal injection of TNF-α causes an accumulation of DC in the DLNs of mice (Cumberbatch and Kimber, 1995) and a decrease in epidermal LC numbers (Cumberbatch *et al.*1994). In addition, UVB induced accumulation of DC in the DLN could be blocked by pre-treatment of mice with neutralising antibodies to TNF-α before irradiation (Moodycliffe *et al.*1994). This evidence in the skin is corroborated in a model of DC migration from the gut to the mesenteric lymph nodes of rats (Macpherson *et al.*1995). It was shown that the injection of 50mg of endotoxin caused an 8-15 fold increase in DC numbers in the lymph between 10-15 hrs after injection. An injection of neutralising antibody directed against TNF-α into these rats prior to injection of the endotoxin abrogated the increased DC migration. However, in contrast to these results, TNF-α has been suggested to act on LC by immobilising them in the epidermis, as an intradermal injection of TNF–α prior to sensitisation prevents hapten induced loss of LC from the epidermis and so blocks the induction of CH (Streilein, 1993). Supporters of this hypothesis argue that TNF-α causes a loss of marker expression (Ia and ATPase) rather than an actual loss of LC. However, the case for TNF-α inducing DC migration is strong. TNF-α fails to downregulate Ia expression on LC *in vitro* (Koch

et al. 1990), and the rapid loss of Ia staining and the presence of a population of Ia positive cells in the epidermis following TNF-α treatment suggests a subset of LC are migrating out of the skin, rather than the induction of the rapid and selective loss of Ia expression by TNF-α (Cumberbatch *et al.* 1994). In addition, the administration of a neutralising polyclonal antibody to TNF-α blocks DC accumulation in DLN of oxazolone or sodium lauryl sulphate (SLS) treated mice, showing that TNF-α is not only involved in the loss of LC from the skin, but also in the accumulation of DC in the DLN (Cumberbatch and Kimber, 1995).

1.3.3 Mechanism of LC migration

Although the mechanism of LC migration is not fully understood, it is likely that adhesion molecules play a role. It has been postulated that the migration signal acts to alter the phenotype of the epidermal LC, causing them to exit from the epidermis. Recent evidence has shown that LC bind to keratinocytes via E-cadherin and that the expression of this molecule is down-regulated on LC during culture (Tang *et al.* 1993) (Table 1.2). Since keratinocytes are the predominant cell population in the epidermis (Todd *et al.* 1993), E-cadherin could tether LC in the epidermis and may influence the morphology of LC in the epidermis. Cytokine signals, which could include TNF-α and GM-CSF, may induce the down-regulation of E-cadherin, weaken adhesive bonds between LC and keratinocytes, and allow migration of LC from the epidermis. ICAM-1, and to a lesser extent LFA-1, may be involved in the migration of LC to local lymph nodes. Intravenous injection of monoclonal antibodies directed against these molecules caused a reduction in the numbers of $FITC^+$, Ia^+ DC found in lymph nodes after FITC skin painting, and an inhibition of the induction of CH (Ma *et al.* 1994). Molecules that have roles in the homing and recirculation of cell populations, are upregulated on LC during culture. There is increased expression of the surface

Surface marker	murine DC	human DC
MHC class I	↑ (spleen and thymic DC) Crowley *et al.* 1989 ↑ (spleen DC) Witmer-Pack *et al.* 1988	↑ (cultured LC) Romani *et al.* 1989
MHC class II	↑ (lymph node DC) Cumberbatch *et al.* 1991 ↑ (cultured LC and spleen DC) Schuler and Steinman, 1985 ↑ (spleen DC) Witmer-Pack *et al.* 1988	↑ (cultured LC) Romani *et al.* 1989
CD45		√ (blood DC) Freudenthal and Steinman, 1990 √ (blood and lymph node DC) Wood *et al.* 1991
ICAM-1	↑ (lymph node DC) Cumberbatch *et al.* 1992	√ (blood and tonsil DC) Hart and Prickett, 1993
ICAM-2		√ (weak on blood and tonsil DC) Hart and Prickett, 1993
ICAM-3		√ Caux *et al.* 1994a
LFA-1	√ (spleen DC) Metlay *et al.* 1990	√ (blood DC) Freudenthal and Steinman, 1990
MAC-1	↓ (cultured LC and spleen DC) Schuler and Steinman, 1985	√ (blood DC) Freudenthal and Steinman, 1990
P150/95	√ (spleen DC) Metlay *et al.* 1990 √ (cultured LC) Agger *et al.* 1990	√ (blood DC) Freudenthal and Steinman, 1990 √ (25% of spleen DC) De Panfilis *et al.* 1989
LFA-3		√ (blood DC) De Panfilis *et al.* 1989

Table 1.2 (i) Phenotype of various DC populations in comparison with freshly isolated LC [↑ expression up-regulated (compared with LC), ↓ expression down-regulated (compared with LC), √ expression, × no expression.

Surface marker	murine DC	human DC
CD1a		× (blood DC) Freudenthal and Steinman, 1990
CD1b		√ (weak on blood DC) Freudenthal and Steinman, 1990
CD1c		× (blood DC) Freudenthal and Steinman, 1990
CD44	↑ (cultured LC) Inaba *et al.* 1994	√ Caux *et al.* 1994a
CD40	↑ (cultured LC) Inaba *et al.* 1994	√ (cultured LC) Romani*et al.* 1989
Fc receptors	↓ (cultured LC) Inaba *et al.* 1994	↓ (cultured LC) Romani*et al.* 1989
CD80	↑ (cultured LC) Inaba *et al.* 1994	↑ (cultured LC) Symington, 1993
CD86	↑ (cultured LC) Inaba *et al.* 1994	√ (CD34+ progenitors cultured with GM-CSF and TNFα) Caux *et al.* 1994b
E-cadherin	↓ (lymph node DC) Borkowski *et al.* 1994	↓ (cultured LC) Blauvelt *et al.* 1995

Table 1.2 (ii) Phenotype of various DC populations in comparison with freshly isolated LC [↑ expression up-regulated (compared with LC), ↓ expression down-regulated (compared with LC), √ expression, × no expression.

molecules CD44 and α4 integrin (the α-chain of LPAM-1/VLA-4) and ICAM-1 (Aiba *et al.*1993). CD44 is a transmembrane glycoprotein with a molecular weight around 85000 (Omary *et al.*1988). Evidence points to this molecule being involved in cell recirculation, by binding specific carbohydrate residues on high endothelial venules (Springer, 1990). VLA-4 has two ligands, VCAM-1 a cell adhesion molecule induced on endothelium by inflammatory mediators, and the extracellular matrix protein fibronectin (Springer, 1990). Adhesive interactions between these molecules on differentiated LC and their ligands may play a role in the migration of LC to the DLN.

1.4. Migration of DC to DLN.

To induce a primary immune response, antigen processed by epidermal cells has to be transported to the local draining lymph node (DLN), where competent antigen-bearing APC can stimulate MHC-restricted proliferation and differentiation of antigen-specific T cell clones. There is good evidence, that will be presented later in this section, that antigen-bearing LC transport antigen to the DLN. Certainly, LC are capable of migration out of the skin; a number of stimuli cause a loss of LC from the epidermis including exposure to low dose UVB light and skin painting with contact sensitisers (Bergstresser *et al.*1980). Depletion of murine epidermal LC using these treatments results in a subsequent accumulation of DC in the lymph nodes draining the treated site (Kinnaird *et al.*1989; Moodycliffe *et al.*1992), which has been attributed to an influx of DC from the skin. However, skin painting with haptens causes an influx of DC into lymph nodes that drain non-sensitised sites, in addition to inducing an increase in DC numbers in DLNs (Hill *et al.*1990). Therefore, some of the increase in DC numbers seen in lymph nodes draining a sensitised or irradiated site, may result from migration of non-LC derived DC into the lymph node.

1.4.1 DC cords in dermis

Whole skin biopsies can be cultured *in vitro* by floating the skin dermal side down on medium, so that the epidermal face exposed to the air. Within 24 h of culture LC in epidermal sheets increased in size and expression of MHC class II molecules, and their numbers were markedly decreased (Larsen *et al.*1990). Explant culture has provided a good source of murine and human DC, since DC migrate out of the skin and can be harvested from the medium after 1-3 days in culture (Larsen *et al.*1990; Pope *et al.*1995). This system has also provided a model to examine the migration of LC out of the skin. After 24 hr of culture, cells with LC morphology were found close to the epidermal-dermal junction, and by 3 days, the dermal lymphatics were filled with strings of LC leaving the skin (Larsen *et al.*1990). T cells also emigrate out of skin organ culture, mostly γδ T cells in mice (Larsen *et al.*1990), while in humans the migrating T cells are mostly αβ TCR with a 'memory' phenotype (Pope *et al.*1995). In both systems DC and T cells migrating out of the skin form clusters. The possible contribution of this to immune responses *in vivo* is still a subject for speculation.

1.4.2 DC in afferent lymphatics

After an appropriate signal, LC migrate into the afferent lymphatics where they are described as veiled cells (due to their long actively moving processes which resemble veils). Following cannulation of the afferent lymphatics of rabbits it was found that large mononuclear cells, many displaying surface veils, accounted for 53% of total cells (Kelly *et al.*1978). In addition, 3% of cells contained Birbeck granules (Kelly *et al.*1978). There seem to be major differences in the estimates of the numbers of DC migrating under normal conditions in the afferent lymph. One paper (Steinman *et al.*1995) reviews evidence that 10^5 DC/hr accumulate from cannulated afferent lymph of an unnamed species and argues that this number of DC cannot be derived from the available pool of epidermal LC. Similarly, studies in the sheep (Dandie *et al.*1994) have shown a background level of around $9x10^4$ LC/hr in afferent lymph.

Following treatment with a chemical carcinogen (7, 12-dimethylbenz(a)anthracene) LC numbers in the afferent lymph peaked at day 5 with 3.7 $x10^7$ LC per hour. Although this number of DC represented migration from $50cm^2$ of carcinogen treated skin, the slow-turnover of LC in the epidermis makes it unlikely that all these DC are epidermally derived. However (Kelly *et al.* 1978) report the collection of around $2x10^4$-$4x10^5$ veiled mononuclear cells/hour from from the afferent lymph of rabbits, since 3% of these cells have Birbeck granules, this means that around 600-12000 LC-derived cells migrate via the afferent lymph of rabbits each hour.

Cells migrating via the afferent lymph already display maturational changes enabling them to efficiently induce antigen specific proliferation. In one study the afferent lymph ducts of calves were cannulated, enabling the afferent lymph veiled cells (ALVC) to be studied (McKeever *et al.* 1992). The ability of the ALVC draining from the site of intradermal antigen challenge [variable surface glycoprotein (VSG) from *Trypanasoma brucei*], to induce peripheral blood mononuclear cell (PBMC) proliferation, was measured in monozygotic bovine twins. It was found that proliferation in PBMC from VSG immunised calves was induced, within 30 minutes of intradermal application of antigens. There was no proliferation of PBMC from VSG naive calves. Similar work has been carried out in sheep, using the protein antigens ovalbumin (OVA) and purified protein derivative (PPD) from *bacillus Calmette Guerin* (Bujdoso *et al.* 1989). There was a marked proliferation of OVA and PPD specific T cell lines when incubated with afferent lymph cells from OVA and PPD challenged animals. Afferent lymph cells collected prior to challenge did not induce significant proliferation in the antigen-specific T cell lines. The stimulation was antigen specific as the afferent lymph cells of OVA challenged sheep did not induce proliferation of PPD specific T cells, and vice-versa. In addition, the ability of ALVC to cluster with primary resting T cells was described, a property which is not shared by non-professional APC and one of the reasons why antigen presentation by DC is essential for the initiation of primary responses.

1.4.3 Differentiation of LC

The changes in the morphology, phenotype and function of DC as they migrate to the DLN are thought to be similar to the changes seen *in vitro* when freshly isolated epidermal LC are cultured. Cultured LC lose the ability to process antigen (section 1.1.2.2) and most lose their Birbeck granules (Streilein *et al.*1990). However, cultured LC are significantly better at stimulating autologous and allogeneic T cell responses than are freshly isolated LC. These changes in function are associated with alterations in the phenotype of LC during their migration from the skin and their differentiation into DC.

The changes in the phenotype of murine and human DC, induced during their migration to the lymph node, or after 3 days culture with keratinocyte derived cytokines is shown in Table 1.2. Several molecules that are essential for antigen-presentation are up-regulated on DC during *in vivo* or *in vitro* maturation. ICAM-1 which may be involved in providing the initial antigen independent interaction between DC and T cells (section 1.5.1), is up-regulated on cultured epidermal LC (Aiba *et al.*1993) and DC isolated from LN have higher levels of ICAM-1 than LC isolated from the epidermis (Cumberbatch *et al.*1992). MHC class I and II are upregulated on cultured LC (Shimada *et al.*1987; Schuler and Steinman, 1985) and DC isolated from the lymph nodes show increased expression compared with epidermal LC (Cumberbatch *et al.*1991). Other molecules such as members of the B7 family of costimulatory molecules that are required to induce the differentiation of naive T cells into functional effector cells, are also up-regulated on DC compared with LC.

The cytokines produced by keratinocytes after treatment with haptens or UVB are important in the differentiation of LC into lymphoid DC. Differentiation of murine LC *in vitro* requires the presence of GM-CSF, produced by contaminating keratinocytes (Witmer-Pack *et al.*1988). Culturing highly purified murine LC in GM-CSF increases their ability to stimulate MLRs and LC purified from bulk epidermal cell cultures, where contaminating keratinocytes provide cytokines, are also good

stimulators of MLR (Heufler *et al.*1988). This ability is not only due to improved viability and increased Ia expression (Heufler *et al.*1988). If LC are cultured in the presence of both GM-CSF and IL-1, a two-fold enhancement in their capacity to stimulate MLR is induced compared with LC cultured in GM-CSF alone (Heufler *et al.*1988). Further evidence of a role for GM-CSF in the differentiation/maturation of LC is provided by the inability of LC from unprimed mice to induce an immune response to a tumour associated antigen, unless the cells are pre-incubated with GM-CSF (Grabbe and Granstein, 1993). Pre-incubation of unprimed LC with IL-1α, TNF-α, IFNγ and TGFβ does not affect their ability to induce a response and, indeed, some combinations of cytokines cause a reduction in the immune response (Grabbe and Granstein, 1993). If murine LC are cultured in TNF-α alone, the viability of the cells is maintained, but they do not mature functionally and are poor stimulators of MLR (Koch *et al.*1990). However, TNF-α does induce LC differentiation, causing the down-regulation of macropinocytosis (Caux *et al.*1994a). Culture of human DC progenitors, $CD34^+$ cells isolated from peripheral blood, in GM-CSF and TNF-α causes their differentiation into cells with DC activity (Caux *et al.*1992). TNF-α synergises with other cytokines, notably GM-CSF (Caux *et al.*1992), IL-1 and IL-4, in the differentiation of human $CD34^+$ haematopoietic cells into dendritic Langerhans cells *in vitro* (Caux *et al.*1992). This function may be ascribed to its ability to induce IL-3 and GM-CSF responsiveness which may be important in the early stages of differentiation of these cells *in vitro* (Caux *et al.*1990). After 12 days of culture with TNF-α and GM-CSF the total cell number and the proportion of $CD1a^+$ cells within the population were increased compared to cells to which only GM-CSF was added (Caux *et al.*1992).

1.4.4 Accumulation of DC in DLN

Although the presence of FITC-bearing DC in the DLN of FITC-sensitised mice is not necessarily a marker for epidermal derivation, it is likely, though still a matter of some contention, that most of these DC cells bind FITC in the skin prior to

their migration to the DLN. Two populations of $FITC^+$ DC have been identified in the DLN after skin painting (Macatonia *et al.*1987), one with low levels of FITC and another intensely fluorescent population. DC bearing high levels of FITC first appeared 2-8 hrs after skin painting and they were the only cells able to induce T cell proliferation. In another study, two populations of $FITC^+$ DC were found in the DLN of mice after skin painting; 75% of these DC expressed the antigen F4/80, a marker of LC but not lymph node DC, which suggests a skin derived population (Kripke *et al.*1990). The remaining 25% did not express F4/80. These cells may represent a resident lymph node population that bind free FITC in the lymph node. These skin-derived DC, which bear high levels of FITC, are required for the induction of FITC-specific CH responses (Macatonia *et al.*1986). Further evidence that lymph node DC are predominantly LC derived comes from a model using a monoclonal antibody to deplete Ia^+ cells in mice (Aberer *et al.*1986). This resulted in the loss of Ia+ cells from the spleen and lymph nodes but not the depletion of LC from the skin. Following skin painting, Ia^+ FITC bearing cells appeared in the lymph nodes that could stimulate a FITC specific hybridoma in an MHC class II restricted manner (Aberer *et al.*1986).

Although FITC rapidly associates with proteins *in vivo*, it has been claimed that FITC can move freely to the lymph node without necessarily binding to LC in the epidermis. After ear painting FITC found in the DLN is associated predominantly with IDC. Therefore, free FITC entering the lymph node would have to bind interdigitating DC with a high affinity and high selectivity. Although FITC is a popular contact sensitiser because it can be visualised, other antigens have also been used to study migration. *Leishmania major (L. major)* amastigotes, administered intradermally, induced migration of DC from the skin to the DLN. DC isolated from these nodes were immunostimulatory for *L. major* specific T cells (Moll, 1993). In other studies in sheep and cattle, the afferent lymph veiled cells (ALVC) draining a site challenged intradermally with antigen were collected and used to induce antigen specific proliferation (Bujdoso *et al.*1989; McKeever *et al.*1992).

The most compelling evidence for epidermal LC migrating to the DLN after antigen challenge, is provided in experiments using athymic nude BALB/c mice grafted with skin from C3H mice (Kripke *et al.*1990). When the BALB/c mice were contact sensitised with FITC through the graft tissue, the cells binding FITC in the DLN were found to be of graft donor phenotype. Isolated FITC binding cells from the DLN of BALB/c mice could induce a CH response in C3H but not in BALB/c mice. In the same study it was shown that at least some of the FITC binding cells found in the DLN contained Birbeck granules, a feature of epidermal LC. Similarly, cells bearing rhodamine B (a fluorescent contact sensitiser) and MHC class II have been detected in murine lymph nodes following sensitisation through human skin grafted on nude mice (Hoefakker *et al.*1995). However the numbers of LC in the human skin were very low and it is impossible to derive the number of human DC per lymph node from the presented data. Taking all the evidence from this section as a whole, it seems likely that most of DC which accumulate in the lymph nodes following contact sensitisation are derived from cutaneous APC.

1.5. The induction of primary immune responses

The veiled cells drain into the lymph node and localise in the paracortex where they are called IDC as their dendritic projections show extensive contact with surrounding cells. The IDC present antigen in a MHC-restricted manner to T cells in the lymph node. T cells which are specific for the antigen / MHC complex and which receive the correct signals from the APC (soluble signals and cell-cell interactions) are induced to proliferate and differentiate.

The requirement for professional APC, and in particular DC, in the induction of primary immune responses has been demonstrated. Transgenic mice were bred that differed in the amounts of I-E MHC expressed on the surface of the major APC populations (DC, B cells and macrophages) (Levin *et al.*1993). These mice were then immunised with an I-E restricted peptide antigen. After eight days the CD4+ T cells

were removed from LN draining the site of immunisation and cultured with the immunising antigen. There was a correlation between the *in vitro* proliferative response of the CD4+ T cells and the percentage of I-E expressing cells in the mouse. However, there was no correlation between I-E expression on B cells or macrophages, and the extent of the CD4 T cell recall response. This evidence is indicative of the requirement for DC in the induction of primary immune responses. In the following two sections the process of antigen presentation will be described and related to the features of DC which make them efficient APC.

1.5.1 Clustering with naive T cells

The ability of DC to initiate primary immune responses can be explained, in part, by their capacity to form stable clusters with resting antigen-specific T cells. Splenic DC from mice form clusters with T cells and B cells *in vitro* in the absence of exogenous antigen (Inaba *et al.*1984), while other APC can only cluster with sensitised T cells (Inaba and Steinman, 1984). Antigen-independent adhesion between T cells and the APC precedes antigen-dependent clustering, and may allow DC to "sample" different T cells (Inaba and Steinman, 1986). *In vitro* assays have shown that clustering occurs prior to, and is essential for, T cell proliferation (Austyn *et al.*1988). The interaction between LFA-1 and its ligands ICAM-1,2,3 may be important in this first stage of antigen presentation. The requirement for ICAM-1 expression on APC has been shown by examining the function of mutant APC from mice which had an 80-95% reduction in ICAM-1 expression (Dang *et al.*1990). The ICAM-1^{low} APC had a greatly impaired ability to present antigen to T cells. Reconstitution of ICAM-1 by transfection of the gene into these cells restored normal antigen presentation. ICAM-1 induces important costimulatory signals through the LFA-1 molecule on T cells (Van Seventer *et al.*1992). The ICAM-1 / LFA-1 interaction is also necessary in the clustering of T lymphocytes with other cells (Lorenz *et al.*1993), and it has been suggested that antigen independent adhesion is the first

step toward recognition of the antigen /MHC complex by T cells (Makgoba *et al.*1989). However, antibodies to LFA-1 fail to block antigen-independent clustering of murine spleen DC although they do block the function of clusters by causing a decrease in cell proliferation and cluster stability (Inaba and Steinman, 1987).

1.5.2 Antigen presentation.

It is generally accepted that functional effector cells are generated from naive T cells only after the cell receives signals through both the antigen restricted TCR/MHC pathway and through separate non-antigen specific costimulatory pathways. One such pathway that seems to be important in a number of systems is mediated by interactions between CD28 and molecules of the B7 family. The up-regulation of adhesion molecules on the surface of DC during their migration to the lymph node, provides DC with the ability to induce the proliferation and differentiation of antigen-specific naive T cells in the paracortex of the draining lymph node. In this section the role of MHC molecules and costimulation via B7 molecules will be discussed. However, other costimulatory pathways have been proposed including signalling via CD2/LFA-3 interactions and through heat stable antigen binding its ligand (Enk and Katz, 1994). In addition, soluble signals mediated by cytokines such as IL-1, IL-2 and IL-6 are important in the activation of T cells (Van Snick, 1990), but will not be covered here.

DC express high levels of MHC class II and class I. Indeed, freshly isolated murine LC express around 2-4x10^5 MHC class II molecules/cell (Witmer-Pack *et al.*1988; Schuler and Steinman, 1985) and MHC class II is up-regulated 5-fold during bulk EC culture (Witmer-Pack *et al.*1988) and around 2-fold in enriched LC cultures (Schuler and Steinman, 1985). Similar levels of up-regulation are seen *in vivo* when the density of MHC class II on DC are compared with that on LC (Cumberbatch *et al.*1991). MHC class I levels on LC in bulk EC suspensions also increase around five-fold during overnight culture though constitutive levels of MHC class I are low on

LC (Witmer-Pack *et al.*1988). Therefore DC entering the lymph nodes following migration from the skin are well equipped to provide signalling via the TCR.

There has been much interest recently in the costimulatory functions of members of the B7 family which are ligands for CD28 and CTLA-4 on T-cells. B7-2 (CD86) expression is found within 24 hrs of activation of human B-cells, while B7-1 (CD80) expression peaks several days later (June *et al.*1994). B7-2 is found on peripheral blood DC (Young *et al.*1992), resting human monocytes and on activated T cells, B cells and NK cells (Azuma *et al.*1993). CD28 is expressed widely on both human and mouse resting T cells, while CTLA-4 expression seems to be limited to activated T cells (June *et al.*1994). While both B7-1 and B7-2 are ligands for CD28 and CTLA-4, *in vivo* interactions may be influenced by the availability of the ligands. B7-2 is found constitutively at low levels on murine epidermal LC, and after 24 hrs in culture there is a dramatic up-regulation in its expression (Inaba *et al.*1994) (Table 1.2). B7-1 is not found on epidermal LC normally, but it is induced (to lower levels than B7-2) during culture (Larsen *et al.*1992; Symington *et al.*1993; Inaba *et al.*1994), and is present on splenic DC (Larsen *et al.*1992).

The up-regulation of B7-1 and B7-2 during LC culture and their expression on lymphoid DC suggests the involvement of keratinocyte-derived cytokines. B7-2 up-regulation can be partially decreased during culture in the presence of an anti-GM-CSF antibody (Inaba *et al.*1994), which may reflect a minor role for GM-CSF in the induction of B7-2. Alternatively the isolated LC may have received a signal to up-regulate B7-2 expression during isolation which then may be difficult to reverse (Inaba *et al.*1994). Lipopolysaccharide does not seem to up-regulate B7-2 on DC although it does increase expression of B7-2 on macrophages and B cells (Hathcock *et al.*1994).

Therefore, both B7-1 and B7-2 are expressed on DC in lymphoid tissue with up-regulation during *in vitro* culture of LC, associated with increased APC activity. Freshly isolated LC are less able to stimulate allo-responses than are cultured LC

(Freeman *et al.*1991; Larsen *et al.*1992; Symington *et al.*1993) and cultured LC induce similar allo-responses to freshly isolated LC when B7-1 and B7-2 interactions are blocked using CTLA4-Ig (Symington *et al.*1993). CTLA-4Ig is a fusion protein with the extracellular portion of CTLA-4 spliced to the constant region of human IgG1 molecule, which acts as a soluble ligand for B7-1 and B7-2 (Lane *et al.*1993). Interestingly, the allo-response induced by fresh or cultured LC could be abrogated using CTLA4-Ig and anti-ICAM-1(Symington *et al.*1993).

There is some evidence that B7-1 signalling induces Th1 type responses, while B7-2 mediates Th2 responses. There has been some confusion in the use of CTLA-4Ig to block signalling through the CD28/B7 pathway. Using this method two groups produced conflicting data, the first group used a model of rat allograft where graft rejection was mediated by infiltrating T cells expressing the Th1 type cytokine IFN-γ and mononuclear cells (Sayegh *et al.*1995). Treatment of these animals with CTLA-4Ig increased graft survival, which was associated not with a reduction in graft infiltrate, but with an increase in Th2 cytokine production (IL-4) by infiltrating T cells. In a separate study a similar method was used to prolong survival of BALB/c mice following infection with *L. Major* (Corry *et al.*1994). BALB/c mice are susceptible to Leishmaniasis due to their development of Th2-type responses following infection. CTLA-4Ig given in the first week of infection halted disease progression and was associated with a reduction in IL-4 and IgE levels (Corry *et al.*1994). Although these two results appear to be conflicting the ability of CTLA-4 to bind both B7-1 and B7-2 may explain why treatment with CTLA-4Ig blocked a Th1 response in the first model and a Th2 response in the second. These models represent highly diverged Th1 and Th2 immune responses that become locked into an immunopathogenic response, because of the ability of each response to negatively regulate the other. In these models it is possible that CTLA-4Ig is acting to block the predominant response allowing a more balanced response. While CTLA-4Ig blocks signal mediated by B7-1 and B7-2, the use of specific monoclonals to these molecules

has allowed their roles in the generation of Th1 and Th2 responses to be dissected more completely. In a mouse model of experimental allergic encephalomyelitis (EAE) in which Th2 T cells are associated with the suppression of disease, blocking B7-1 signals during the initiation of disease reduced subsequent symptoms, while blocking signalling through the B7-2 pathway increased disease severity (Kuchroo *et al.* 1995). Blockade of the B7-1 pathway was associated with the generation of Th2 type T cell clones, while Th-1 T cell clones were generated following blockade of B7-2 signalling. This evidence suggests that B7-1 preferentially induces Th1 type responses, with B7-2 reciprocating by favouring the induction of Th2 responses.

The above results may help to explain the disparate effects on accessory cell activity induced by the blockade of B7-1 (CD80) and B7-2 (CD86) activity. The ability of human DC, obtained by culturing $CD34^+$ peripheral blood progenitor cells with GM-CSF and TNF-α, to induce allo-responses was inhibited by monoclonal antibodies against CD28 (Caux *et al.* 1994b). However, though monoclonal antibodies against CD80 had little effect on the allo-response, monoclonal antibodies against CD86 suppressed the allo-response by 70% (Caux *et al.* 1994b). When used together monoclonal antibodies to CD80 and CD86 caused a 90% reduction in allo-response (Caux *et al.* 1994b). CTLA4-Ig binding was completely inhibited in the presence of monoclonals to CD80 and CD86, suggesting that there is not a third ligand for CTLA-4 on DC.

Due to the these and other changes in phenotype summarised in Table 1.2, IDC and cultured LC become specialised at presenting antigen to T cells, and are efficient at activating unprimed LNC and in the induction of alloresponses *in vitro* (Streilein and Grammer, 1989).

1.5.3 Production of a cutaneous immune response.

T cells that recognise, and bind to, the specific antigen/MHC complex on the IDC and receive the necessary costimulatory signals, are induced to proliferate.

Activation signals also induce differentiation of the T cells which undergo changes in both morphology and phenotype. These T cells exit the lymph node in the efferent lymph and enter the bloodstream via the thoracic duct. Recently activated $CD45RO^+$ T cells, the so called memory subset, have been shown to exhibit specialised recirculation patterns. In the sheep the loss of L-selectin on these cells means that they are less likely to recirculate to peripheral lymph nodes and instead they circulate preferentially through tissue sites including the skin (Mackay *et al.*1992). Once at the site they orchestrate adaptive immunity, inducing responses such as CH and DH, whose effector mechanisms were described in section 1.0.3.1.

1.5.4 Secondary cutaneous responses.

The antigen presenting pathway that has been described, LC processing antigen in the periphery and presentation to specific T cells in the DLN, is relevant to all cutaneous immune responses. However, this mechanism may be augmented by alternative antigen presentation pathways in a secondary response, where there will be a pool of activated or semi-activated T cells specific for the antigen. It was noted in the previous section that sub-populations of these cells show preferential recirculation to tissue sites including the skin. Therefore in a secondary response, T cells could encounter antigen in the periphery bound to LC or other antigen presenting cell populations such as B cells, macrophages and keratinocytes. Memory/activated T cells expressing CD45RO can be activated by a wide range of APC, while activation of naive T cells requires dendritic cells (Bradley *et al.*1993). Therefore skin homing populations of T cells, after being presented with antigen in the periphery, could release inflammatory factors that initiate a cascade response leading to the migration of effector cells through the endothelium into the skin.

1.6. Ultraviolet light

1.6.1 Sources of UVB

Ultraviolet light comprises wavelengths of electromagnetic radiation between 200-400nm. The spectrum of UV light is divided in to 3 arbitrary units UVA, UVB and UVC. The longest wavelengths (315-400nm) lie within the UVA range. These are present at the highest intensity in terrestrial sunlight. Light in the UVB range (290-315nm) is present in sunlight at a lower intensity than UVA (100-1000 times less) but UVB wavelengths have the largest biological effects per unit of irradiation. The shortest wavelengths of UV light, UVC (200-290nm), would be very damaging to biological systems, but fortunately are efficiently blocked by atmospheric ozone.

There are a number of artificial sources of UVB light, which differ in their outputs. In this study the Philips TL-20W/12 was used as a source of broadband UVB. These bulbs emit a broad-band of UV with peak output in the UVB range. However, there is some output in the UVC and UVA ranges. In addition a TL01 lamp, which emits predominantly at 311-312nm, was used in the chronic UVB exposure study. The output spectra for both sources are presented in Figure 1.2.

As the surface of the earth is likely to become more exposed to UVB radiation with a decrease in the protective ozone layer, concern is growing about the impact of this change on animal and plant ecosystems.

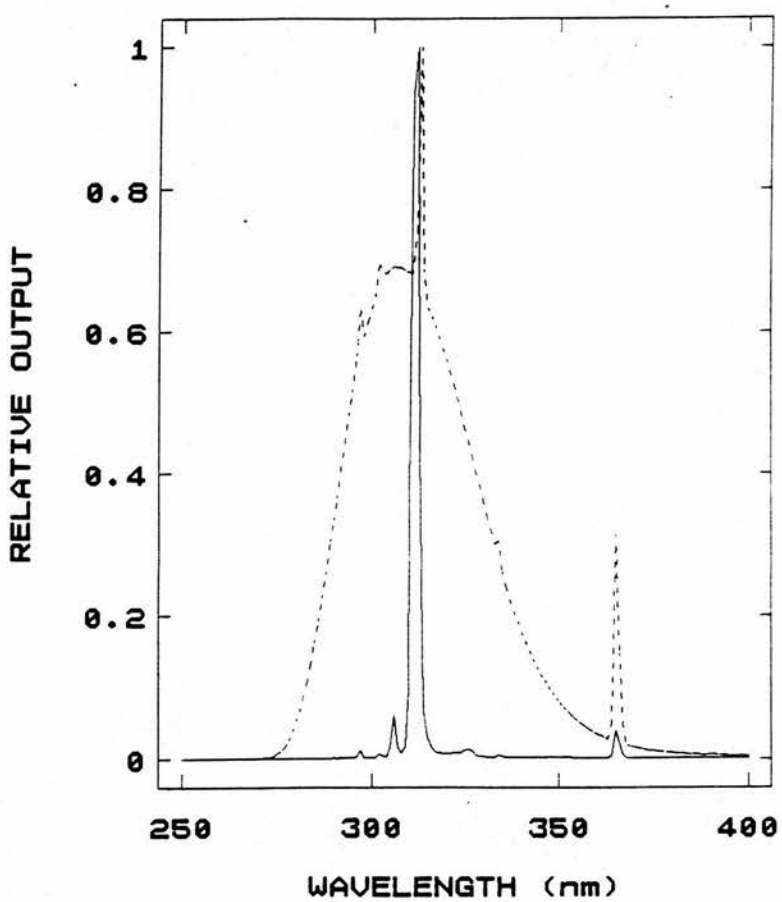

Figure 1.2 The emission spectra for the broadband UVB lamp (TL12 dotted line) and the narrowband TL01 lamp (solid line). Figure reproduced from Moodycliffe *et al.* 1994.

1.7. UVB and immunosuppression

1.7.1 UVB: Role in the induction and promotion of tumours

1.7.1.1 Epidemiology of skin cancer

The incidence of all skin cancers (melanoma and non-melanoma) has been rising for the last 20 years. The epidemiological evidence suggests an association between exposure to sunlight and the development of all types of cutaneous malignancy. The most direct relationship is seen in the development of squamous cell carcinomas (SCC) which result from accumulated sun exposure. Therefore these cancers are seen more often in people living in sunny areas of the world and appear at chronically exposed body sites e.g. the head, neck and backs of hands. There is also a weaker relationship between accumulated sun exposure and the incidence of basal cell carcinomas (BCC) (de Gruijl, 1993). BCC appear mainly on the face and neck but rarely on the backs of hands; additionally 10% of these cancers are found on the trunk (de Gruijl, 1993). There is no strong evidence to link the development of melanomas with accumulated exposure. Immunocompetent hairless mice, chronically exposed to UVB, never develop melanoma, and outdoor workers are less at risk from melanoma than urban white collar workers (de Gruijl, 1993). Epidemiological evidence supports the connection of childhood sunburn with melanoma development in later life. However this relies on the recall of 'burning episodes' by patients with melanoma (de Gruijl, 1993)

1.7.1.2 UVB and tumour induction

With regard to the induction of cancer, UVB has received the most attention because the peak carcinogenicity per unit of ultraviolet exposure (J/m^2) occurs at 293nm. Also the action spectrum for carcinogenicity in mice closely follows the action spectrum for erythema which is induced most potently by the UVB component of sunlight. However, more interest is being shown in the role of UVA because,

although carcinogenicity drops by a factor of 10^4 for wavelengths over 340nm (de Gruijl, 1993), the high intensity of UVA in sunlight and the inability of certain sunscreens to block UVA wavelengths means that individuals could be exposed to high doses. However, in mice UVA has a protective effect, delaying the appearance of tumours induced by chronic UVB exposure (Bech-Thompsen *et al.*1977). This protective effect of co-exposing mice to UVB and UVA together was seen at all but the highest dose of UVA (45.6 kJ/m^2 daily). There has been little or no attention given to UVC because although UVC wavelengths are carcinogenic, they are blocked efficiently by atmospheric ozone and so are unlikely to be of importance in terrestrial sunlight.

UV light is absorbed by a number of biological proteins, the most critical of which is DNA. The role of DNA damage in UVB-induced immunosuppression will be discussed in section 1.8.2.1. However, the importance of UVB as an initiator of tumourigenesis may result from its ability to cause mutations in the p53 gene affecting the ability of this locus to control the cell cycle. Cells that lack functional p53 are unable to arrest in the G1 phase of their cycle. G1 arrest is required to institute DNA repair mechanisms, and therefore cells with mutant p53 accumulate DNA damage with an increased chance of transformation. Photodamage caused by UVB has been shown in the p53 gene in all skin cancer types.

1.7.1.3 UVB and tumour promotion

The spontaneous regression of melanomas in occasional patients and the development of a variety of cancers, mainly cutaneous cancers and lymphomas, in immunosuppressed individuals has led to the acceptance that the immune system is involved in the control of certain tumours, particularly in the skin. Inate (natural killer cells, lymphokine activated killer cells and proinflammatory cytokines) and adaptive (T and B cell) mechanisms are thought to be involved in immunity to tumours. The role of the SIS in tumour control may be to monitor the cells in the skin for changes

associated with transformation, and to destroy any transformed cells that are found (tumour surveillance). If such tumour surveillance exists, the outgrowth of a tumour from a transformed cell would require either a weak immune response, or the ability of a transformed cell or tumour to avoid or outgrow the immunological response. Initial experiments showing that UVB light weakened the immune response to tumours, and that UVB was acting as a tumour promotor, resulted from work on SCC induced by chronic irradiation of mice (Fisher and Kripke, 1977). These SCC were immunogenic, being rapidly rejected after transplantation into syngeneic recipients, but were not rejected in syngeneic recipients that had been pre-irradiated. Therefore as well as initiating tumours by causing damage to DNA, UVB may promote of tumour growth by inhibiting tumour surveillance.

The experimental evidence that UVB decreases anti-tumour immune responses is corroborated by clinical studies in which a clear link between susceptibility to UVB-induced immunosuppression and the development of all forms of cutaneous carcinoma has been found. Over 90% of individuals with a previous history of cutaneous neoplasms show susceptibility to UVB, as indicated by suppressed CH response following UVB, compared with 40% of the general population (Streilein *et al.* 1994). Assuming both groups have been exposed to similar levels of UVB-induced DNA damage, the fact that UVB-resistant individuals are barely represented in the cancer group, infers that UVB-resistance is an advantage in coping with the damaging effects of UVB on DNA. The loss of tumour immunity, during the immunosuppression that follows exposure to UVB in certain individuals, may allow transformed cells to escape immune control, accumulate further mutations, and become cancerous.

1.7.2 Suppression of immune responses to infectious disease

Because of the ability of low-doses of UVB to induce suppression of cell-mediated immunity, modulation of immune responses to infectious agents may also occur. There is now evidence from a number of rodent models that UVB can affect

immune responses to viruses, bacteria, fungi and parasites. One virus that has received attention in this area is herpes simplex virus-1 (HSV-1). It infects the epithelial cells of the skin causing local lesions and persists in the ganglia in a latent form. It can recrudesce at intervals and exposure to sunlight is one common stimulus for reappearence of lesions (Norval, 1992). In a mouse model it has been shown that a low-dose of UVB prior to infection with HSV induces suppression of DH to the virus when the mice are subsequently challenged (Howie *et al.*1987) (see section 1.8.2.2). The suppression of responses in this model was associated with the generation of splenic T cells which transfered suppression of DH to naive recipients. UVB may be of importance principally in reactivating the latent virus. However, it is possible that reduced immune responses to HSV which follow exposure to UVB provide a more permissive environment for viral replication, thus converting a non-clinical infection into an overt one.

The role of UVB in the progression of disease in HIV^+ individuals has been studied, not only because of the concern that UVB may affect responses to the virus, but also because it may increase susceptibility to the opportunistic pathogens that infect immunocompromised individuals. Interestingly, low-doses of UVB ($400J/m^2$) activate the human HIV promoter causing the transcription of HIV within infected cells (Miller *et al.*1992). Since the virus has been detected in the skin of HIV^+ individuals and LC have been proposed as a reservoir of infection (Weber and Weiss, 1988), it is possible that UVB may directly increase the titre of virus by activating transcription within cells in the skin. In addition to its ability to promote viral transcription, the immunosuppression caused by UVB may well exacerbate the suppression caused by HIV. This is certainly true in the mouse model of AIDS (MAIDS) where exposure to low-doses of UVB was shown to enhance the immunosuppression and the progression of the disease (Brozek *et al.*1992). Because of these effects there have been warnings that HIV^+ individuals, and other immunocompromised individuals should avoid sun-exposure. There has been an interesting study examining the

mechanism of immunosuppression in Rauscher leukaemia virus (RLV) infected mice (Gabrilovich et al. 1994). Following intravenous infection RVL DNA was detected in Langerhans cells and lymph node DC. CH responses were suppressed in infected mice. Suppression was associated with a reduction in LC accessory function in MLR. In addition, the suppression reflected an inability of LC to migrate from the skin following epicutaneous FITC application and an accompanying lack in DC accumulation in lymph nodes draining the sensitised sites (Gabrilovich et al. 1994). Since HIV and RLV are retroviruses and both infect LC, it would be interesting to speculate whether HIV and RLV cause similar effects on cutaneous immunity. If this is the case, then it may help explain the inability of symptomatic HIV^+ individuals to control the growth of warts and cutaneous neoplasms.

There have also been a number of studies that have reported suppression of immune responses to bacterial infections. In an experimental model, the causative agent of Lyme disease *Borrelia burgdorferi* (Bb), was used to examine the effect of UVB on both DH and humoral responses in mice (Brown *et al.*1995). The proposed ability of UVB to cause a switch from a Th1 to Th2 response (section 1.8.3.2) has been hypothesised to be responsible for UVB-induced suppression of immune responses in a number of disease states. In this model exposure to a single high-dose of UVB ($10kJ/m^2$) 4 days prior to immunisation suppressed DH responses to Bb (Brown *et al.*1995). The role of a Th2 switch in this suppression was supported by evidence that IL-10, a Th2-type cytokine, was important in the suppression of DH responses following UVB. UVB and IL-10 also suppressed the production of IgG2a and 2b antibodies that are associated with Th1 responses (Brown *et al.*1995). In another study, DH responses to the mycobacterium *Bacillus Calmette-Guerin* (BCG) were unaffected following subcutaneous inoculation through skin exposed to 400 J/m^2 UVB for 4 consecutive days (Jeevan *et al.*1992). However, the course of BCG infection in UVB treated mice was different, with greater numbers of mycobacterium in the lymph nodes and the infection taking longer to clear.

Systemic fungal infections are a problem associated mainly with immunocompromised individuals. The only fungal pathogen that has been studied, *Candida albicans* (*C. albicans*), causes infections of the mucous membranes but in compromised individuals can cause systemic disease. The DH response to *C.albicans* is reduced if a single dose of UVB (46.8 kJ/m^2) is given immediately before or after sensitisation (Denkins *et al.*1989). In a model of lethal *C.albicans* infection, immunisation of mice with formaldehyde-fixed *C.albicans* 6 days prior to i.v. challenge of mice with a lethal dose of the fungus increased the LD50 from 15 days to over 25 days (Denkins and Kripke, 1993). Exposure to UVB prior to sensitisation reduced DH responses, but did not affect survival, while exposure to UVB 1 day before challenge reduced survival time by 50% (Denkins and Kripke, 1993). However, in both these studies the high dose of UVB, approximately 31 minimal erythemal doses (MED), required to affect DH responses and the course of disease, suggests UVB may have a limited impact on *C. albicans* infection following environmental exposure.

The parasitic infections which have been studied most frequently are *L. Major*, and *Schistosoma mansoni* (*S.mansoni*) in mice models and the worm *Trichinella spiralis* (*T.spiralis*) in the rat. In *L. major* infections, chronic low-dose UVB reduced the size of lesions but increased the numbers of animals that died from the disease, while acute low-dose UVB failed to affect infection with *S.mansoni* (Jeevan *et al.*1995). Exposure of rats to UVB reduced their resistance to *T. Spiralis* infection (Goettsch *et al.*1994).

1.7.3 Suppression of CH and DH responses

The suppression of CH responses by UVB is a well characterised phenomenon in mice (Toews *et al.*1980; Moodycliffe *et al.*1994; Schimizu and Streilein, 1994) and in humans (Tie *et al.*1995; Cooper *et al.*1992). Early experiments showed that exposure to sub-erythemal doses of UVB on four consecutive days induced a decrease

in the number of epidermal Langerhans cells in exposed skin (Toews *et al.*1980), and that these sites were unable to support the induction of contact hypersensitivity, a property shared by skin sites that are naturally deficient in Langerhans cells (Toews *et al.*1980). This same study elucidated a number of features of UVB-induced suppression of CH that are relevant. Firstly, the induction of immunosuppression immediately following low dose (400 J/m^2 for 4 conscecutive days) UVB exposure was confined to the irradiated site (local immunosuppression). Secondly, mice were not simply unresponsive to DNFB, the sensitising chemical, following exposure through LC-deficient skin but showed antigen specific tolerance. Unresponsiveness to an antigen would imply the immune system had failed to recognize the antigen. However, since mice which were first sensitised through LC-deficient skin were still unable to respond to DNCB, but not other unrelated sensitisers such as oxazolone, following resensitisation through an unirradiated skin site 14 days later, it is obvious that the suppression of CH responses in these mice is not due simply to unresponsiveness, but to an active tolerance. Similarly in humans, UVB can suppress CH responses to DNFB in certain individuals and results in long-term (up to 4 months) tolerance to the sensitiser in around 30% of people who were exposed to the chemical through irradiated skin (Cooper *et al.*1992). Another important feature of UVB-induced immunosuppression following exposure to low doses and high doses of UVB is the induction of a T cell population in the spleen that transfers reduced CH responses to naive syngeneic recipients, so called 'suppressor T cells'(Elmets *et al.*1983; Glass *et al.*1990). However, while these suppressor cells are found in all strains of mice following UVB irradiation (Glass *et al.*1990), only certain strains actually show suppressed CH responses in response to UVB (Streilein and Bergstresser, 1988; Noonan and Hoffman, 1994).

UVB-induced suppression of DH responses to a number of agents including HSV (Howie *et al.*1987) *and C. albicans* (Denkins and Kripke, 1993) has been demonstrated. DH responses are different in a number of respects from CH responses

(section 1.0.3) and generally they require lower doses of UVB to induce suppression. A single dose of 960 J/m^2 3 days prior to infection with HSV caused a 50-90% suppression in DH response (Howie *et al.*1987), while in CH responses two doses of 1440J/m^2 were required to suppress the elicitation response to the same extent (Moodycliffe *et al.*1994). Similarly, Kripke's group, which use 10kJ/m^2 to cause systemic suppression of CH responses, use 5kJ/m^2 to suppress DH responses to *C.albicans* (Strickland *et al.*1994). Like CH responses, the suppression of DH is associated with the induction of a T cell population in the spleen that transfers immunosuppression of DH responses (Howie *et al.*1987)

1.7.4 Local and systemic immunosuppression

The phenomenom of UVB-induced immunosuppression is complicated further by evidence that UVB causes not only local suppression to agents sensitised through irradiated sites, but also, under certain conditions causes systemic immunosuppression. Though initially thought to be dependent on UVB dosage with high-doses being associated with systemic immunosuppression, the main determinant of whether UVB has local or systemic effects seems to be the time between irradiation and sensitisation. It has been shown that UVB-dose responses for local and systemic immunosuppression are identical (Noonan and De Fabo, 1990). Therefore, irrespective of dosage, suppression was confined to the irradiated site immediately following irradiation and up to 2 days later. However, if sensitisation was delayed until 3 days after the last irradiation CH responses were suppressed whether they were induced at local or distant sites (Shimizu and Streilein, 1994; Noonan and De Fabo, 1990; Kripke and Morrison, 1986). The mediators of local and systemic immunosuppression may be different. Thus, treatment of mice with antiserum to TNF-α failed to abrogate systemic UVB-induced CH impairment (Shimizu and Streilein, 1994), but protected against local CH impairment (Moodycliffe *et al.*1994). A number of mediators of systemic immunosuppression have been suggested,

including *cis*- UCA and IL-10. The role of these mediators in UVB-induced immunosuppression will be discussed in sections 1.8.2.2 and 1.8.3.2 respectively, and TNF-α in section 1.8.3.1.

1.7.5 Strain differences in UVB induced immune suppression

Strain differences in the susceptibility to UVB induced suppression of CH responses have been reported in mice (Streilein and Bergstresser, 1988; Noonan and Hoffman, 1994) and appear to be similar in humans (Tie *et al.*1995; Cooper *et al.*1992). In both mice and man, the use of differing protocols results in some anomalies which will be discussed. In mice two main methods have been used; a model of local immmunosuppression where the mice are sensitised immediately following irradiation on the exposed site (Streilein and Bergstresser, 1988), and a model of systemic immunosuppression in which mice are sensitised 3 days after irradiation on an unexposed site (Noonan and Hoffman, 1994). In the first model (Streilein and Bergstresser, 1988), a number of inbred mice received 150-600 J/m^2 UVB for 4 days on their shaved backs prior to sensitisation on the same site. Two groups were identified; UVB susceptible strains (C57BL/6, C57BL/10 and C3H/HeN strains) that showed over 80% suppression of CH responses following UVB, and resistant mice (A/J, BALB/c and DBA/2 strains) which showed less than 10% suppression. Results from crosses of resistant and susceptible animals suggested that at least two independent loci were involved in the trait and experiments using congenic animals that differed only at their MHC loci suggested that the H-2^d haplotype conferred resistance (Streilein and Bergstresser, 1988). The genetic basis for UVB-susceptibility was of continued interest and later studies by the same group suggested the involvement of two loci, the *lps* locus which codes for lipopolysaccharide (LPS) responsiveness in mice and the *tnfa* locus which is contained within the H-2 region on chromosome 17 (Yoshikawa and Streilein, 1990; Vincek *et al.*1993). The *lps* locus was proposed because of the differing susceptibility of C3H/HeN and C3H/HeJ mice

to UVB (around 90% and 50% suppression of CH responses respectively). These strains differ only at the *lps* locus. The expression of lps^n by C3H/HeN, which makes these mice responsive to the effects of LPS (lethality, B cell activation and pro-inflammatory cytokine secretion by macrophages) also makes them more susceptible to the effects of UVB. In contrast, the expression of lps^d by C3H/HeJ mice makes them less responsive to endotoxin and partially protects them against the immunosuppressive effects of UVB (Yoshikawa and Streilein, 1990).

Although the proposal that differences in the *tnfα* locus confer resistance or susceptibility to UVB-induced immunosuppression is widely accepted, the evidence is not decisive and the mechanisms speculative. The initial indication was the identification of differing H-2 loci as being important in strain differences in responses to UVB (Streilein and Bergstresser, 1988). C3H/HeN and C3H/HeJ mice were examined for their response to TNF- α. Locally administered TNF-α suppressed CH responses in both strains (see section 1.8.3.1), but there was some evidence that C3H/HeN mice were more susceptible than C3H/HeJ mice to TNF-α mediated suppression of CH (Yoshikawa and Streilein, 1990). By analysing the sequence of the *tnfα* gene in UVB-resistant and suseptible mice, a polymorphism at position 329 was identified (a C-T change in UVB susceptible strains) and a minirepeat was found in the 5' untranslated region of the *tnfα* locus in resistant but not susceptible strains (Vincek *et al.* 1993). The relevance of these polymorphisms is uncertain. However, the *tnfα* locus seems to be unimportant in systemic UVB-induced immunosuppression (Noonan and Hoffman, 1994) as the model which is described in the paragraph below failed to associate differences in H-2 haplotype with responses to UVB.

The results from the systemic model in which trinitrochlorobenzene (TNCB) was used as a sensitiser (Noonan and Hoffman, 1994) were similar in many respects to those shown in the local model (Yoshikawa and Streilein, 1990). Three subsets of mice were reported; a high susceptibility group (50% suppression with 0.7-2.3 kJ/m^2) which included C57BL/6 and C57BL/10 mice, a group showing intermediate

susceptibility (50% suppression with 4.7-6.9 kJ/m^2) which included DBA/2, C57BR, C3H/HeN, C3H/HeJ, CBA/N and A/J mice, and a low susceptibility group (50% suppression with 9.6-12.3 kJ/m^2) which included BALB/c, AKR, SJL and NZW mice. Comparison of the results from the local and systemic studies showed some notable differences which may result from the differing protocols used. The most interesting difference is that the C3H/HeN and C3H/HeJ strains each show similar dose responses to UVB, suggesting that the *lps* locus is not involved in systemic UVB-induced immunosuppression. The second difference between the results is in the status of DBA/2 mice which were resistant in the local study (Yoshikawa and Streilein, 1990) but intermediately susceptible in the systemic study (Noonan and Hoffman, 1994).

Just as inbred mice show differing susceptibilities to UVB-induced immunosuppression, outbred human populations seem to show a similar pattern. Around 40% of the total population show a suppression of CH responses following exposure to a low-dose UVB regimen (4x1440 J/m^2) (Tie *et al.*1995). Interestingly, susceptibility to UVB-induced immunosuppression is a risk factor in the development of skin cancers (section 1.7.1.3). However, as with mice, the data are complicated in so far as differing protocols have been used by investigators . In a separate study (Cooper *et al.*1992), exposure to 0.75-4 MED of UVB suppressed CH responses to DNCB in most individuals. Only at the lowest dose of UVB was there a population (31%) of people who were sensitised successfully. However, the concentrations of DNFB used to sensitise the subjects were very different; 30μg (Cooper *et al.*1992) compared with 2mg (Tie *et al.*1995), and, without knowing the MEDs in both studies, it is difficult to compare the levels of radiation given.

1.8 Mechanisms of UVB-induced immunosuppression.

1.8.1 UVB and cutaneous APC

1.8.1.1 UVB and LC

For the last 16 years, since UVB was first shown to deplete LC from the skin (Toews et al.1980), there has been much interest in these cells as mediators of immunosuppression. UVB induces the depletion of LC from both UVB sensitive and UVB resistant strains of mice (Streilein and Bergstresser, 1988). Exposing mice to 100 J/m^2 of UVB on 4 consecutive days caused LC numbers to drop from between 750-1100 LC/mm^2 prior to irradiation, to between 50-250 LC/mm^2 immediately following the final dose (Streilein and Bergstresser, 1988). Generally, slightly more LC remained in the skin of resistant mice following UVB treatment, though the differences were small with a mean of 75 LC/mm^2 in sensitive strains and 193 LC/mm^2 in resistant mice following UVB. In addition to the depletion, those LC that remained in the skin showed altered morphology, with a loss or attenuation of dendritic processes (Toews et al.1980; Streilein and Bergstresser, 1988).

As already mentioned (section 1.7.3), the application of haptens through LC-deficient skin results in the generation of antigen-specific tolerance to the hapten, suggesting that the suppression of CH may be directly related to loss of the epidermal LC. However the loss of LC does not fully explain UVB-induced immunosuppression. Firstly, UVB resistant mice are still able to induce hapten responses through LC depleted skin. Secondly, it seems that it is not a lack of antigen presentation, but altered antigen presentation that results in suppressed immune responses following UVB. A loss or lessening of an immune response to a hapten would account for the suppression seen after UVB, but does not explain why hapten-specific immunosuppression is still found in mice after a secondary exposure to the hapten via an unirradiated skin site (Toews *et al.*1980). This generation of hapten-specific tolerance following UVB suggests the alteration of, rather than the lack of, an

immune response. Tolerance generated in this way is associated with the induction of regulatory/suppressor T cells which can transfer suppression of CH (Elmets *et al.*1983) and DH responses (Howie *et al.*1987). Finally, the ability of UVB to generate both local and systemic immunosuppression, suggests that in some cases UVB influences immune responses without acting directly on the APC.

The effect of UVB on LC function

In view of the difficulties of reconciling the disparate effects of UVB exposure and LC numbers in the skin, a number of studies have examined LC function directly. Thus, irradiation of human epidermal cell suspensions (8-20% LC) and purified LC populations (70-90% LC) with 100-200J/m^2 of UVB *in vitro* inhibits significantly their ability to stimulate primary alloresponses, mitogen-induced proliferation and proliferative responses to recall antigens (Rattis *et al.*1995). Similarly, exposure of murine epidermal cell suspensions to a low dose of UVB (25 J/m^2) inhibits anti-CD3 induced proliferation of T cells, while 100-200 J/m^2 abrogates completely their stimulatory capacity (Tang and Udey, 1991). It has also been demonstrated *in vitro* that UVB (50-100 J/m^2) inhibits the up-regulation of intercellular adhesion molecule-1 (ICAM-1) expression by LC a molecule that is normally up-regulated during culture (Tang and Udey, 1991) or during the migration of LC to the DLN following skin sensitisation (Cumberbatch *et al.*1992). However, *in vitro* exposure of LC to 100 J/m^2 of UVB is cytotoxic to LC, causing their eventual loss from cultures after 48-72 hrs (Tang and Udey, 1992).

Other evidence suggests that UVB-irradiated LC lose their ability to stimulate T helper 1 (Th1) cells, a $CD4^+$ T cell subset that has been identified in mouse and man (Mosmann and Moore, 1996; Romagnani, 1991) . The concept of Th1 and Th2 immunity will be discussed in greater detail in section 1.8.3.2, but briefly the secretion of IL-2 and IFN-γ by Th1 cells make them potent activators of cell-mediated inflammatory responses. In contrast, the cytokines secreted by Th2 cells (IL-4, 5, 6 and 10) favour the production of humoral immune responses. LC were purified from

epidermal cell suspensions and exposed to 200 J/m^2 of UVB. When these cells were used to present keyhole limpet haemocyanin (KLH) to Th1 T cell clones specific for the antigen, the irradiated LC induced long-term tolerance (up to 16 days) (Simon *et al.*1991). Using the same system it was shown that the ability of UVB-irradiated LC to induce antigen specific tolerance was restricted to Th1 cells and that the irradiated LC were capable of activating Th2 cells (Simon *et al.*1992)

All the evidence presented so far has been generated in *in vitro* systems, but the action of UVB on skin and cell suspensions is likely to be different and difficult to compare. However, *in vivo* studies have suggested a functional change in APC function following UVB by assay of cytokines produced by LNC from normal and irradiated mice (Simon *et al.*1994). Mice were exposed to four consecutive doses of 200 J/m^2 of broadband UVB followed by skin painting with DNFB on the day of the last irradiation and 24 hrs later. Treatment with UVB suppressed CH responses, and the LNC proliferative response to DNBS *in vitro*. In addition LNC from UVB irradiated mice produced lower levels of the Th1 cytokines IL-2 and IFN-γ, following stimulation with the antigen *in vitro* .

1.8.1.2 DDC and macrophages

Secondary antigen-presentation pathways have also been proposed as an explanation for the UVB resistance found in certain strains of mice. It has been suggested that UV resistant mice possess a second antigen presentation pathway, separate from epidermal LC, possibly mediated by DC in the dermis (Streilein, 1989). Tape stripped skin which is selectively depleted of epidermal cells, was able to support CH in UVB resistant but not UVB sensitive mice. However surgical excision of hapten painted skin within 1 hr of application, which removes both the dermis and epidermis, prevented the induction of contact hypersensitivity. Recently, it has been shown that dermal cells, haptenated *in vitro*, from UVB-exposed UV-resistant mice can transfer CH responses to naive mice. In contrast haptenated dermal cells from

UVB-exposed UV-susceptible strains were unable to transfer CH and instead induced tolerance (Kurimoto *et al.*1994). These results suggest that in resistant strains the dermal populations are able to provide a secondary antigen presenting pathway, while in susceptible strains these cells induce tolerance.

MHC class II$^+$ APC have been identified in the dermis of mice. Around 2% of cells in murine dermal cell suspensions normally express MHC class II (Kurimoto *et al.*1994). This population contains MHC class II$^+$ LC-like interstitial dendritic APC and dermal macrophages (Duraiswamy *et al.*1994). The LC-like population has APC function, with the dermal macrophage population having no accessory function in syngeneic MLR. However, LC-like cells were found in the perivascular and interstitial dermis of resistant and susceptible strains (Duraiswamy *et al.*1994), so UVB susceptibility cannot be attributed to a lack of antigen presentation by this population.

Following UVB irradiation there is an alteration in the balance of APC populations with the loss of LC from the epidermis accompanied by an increase in macrophage populations in the dermis. Exposure to UVB results in the expansion of the CD36$^+$ subset of MHC class II$^+$ dermal cells which rises from 17 to around 40% of total dermal DR$^+$ cells by day 3 after exposure (Meunier *et al.*1995). Neutrophils (MHC class II$^-$, CD11b$^+$, GR-1$^+$), macrophage-like APC (MHC class II^{hi}, CD11b$^+$) and macrophages (MHC class II^{lo}, CD11b$^+$) also infiltrate into the murine epidermis 3 days after exposure of BALB/c mice to relatively high doses of UVB (Cooper *et al.*1993) and after low doses (1380 J/m^2) in C3H/HeN mice (Hammerberg *et al.*1994) and may be involved in tolerance induction. The role of these cells in tolerance induction was shown by transferring hapten derivatised EC from irradiated and unirradiated mice, into naive syngeneic recipients. Tolerance was only transferable with EC from mice treated 3 days earlier with UVB, a time when the leukocyte infiltrate was maximal (Hammerberg *et al.*1994). By depleting 3 day UV-EC cell suspensions with an isotype control or a rat-anti mouse CD11b or MHC class II

monoclonal antibody, it was shown that macrophages were responsible for the induction of tolerance (Hammerberg *et al.* 1994). Macrophages also appear in the human epidermis during the elicitation phase of the CH response, are $CD1^-$ $OKM5^+$ (CD36 monocyte/platelet marker) DR^+ (Baadsgaard *et al.* 1990). They were found not to suppress the CH response and were shown to be responsible for up to 50% of antigen presentation capacity during hypersensitivity responses (Baadsgaard *et al.* 1990). However, a cell population with the same phenotype, which is found in the epidermis after irradiation by certain wavelengths of UV light (UVB and UVC) (Baadsgaard *et al.* 1987), activates a suppressor T cell population (Baadsgaard *et al.* 1988). In another study, UVB induced a loss of cells with LC markers and the appearance of macrophages that may have antigen-presenting activity (Cooper *et al.* 1986).

The mechanism by which infiltrating macrophages induce tolerance still remains unclear. It has been reported that macrophages represent the main source of IL-10 in human EC suspensions following UVB exposure (Kang *et al.* 1994). Presumably, the induction of suppressor cell circuits by macrophages (Baadsgaard *et al.* 1988) would require their migration to DLN. There has been little work in this area, although an increased proportion of macrophages in lymph nodes draining irradiated skin has been reported (Bucana *et al.* 1994). While the precise mechanism of macrophage induced tolerance is unclear, the alteration in dermal and epidermal APC populations may be responsible for the local immunosuppressive effects induced by UVB. The differences in these populations following exposure of UVB sensitive and resistant mice has still to be determined.

1.8.2 UVB photoreceptors

1.8.2.1 Induction of UVB induced suppression by DNA damage

UVB has been described as a complete carcinogen because of its ability to damage DNA and suppress immune responses to tumours, which means it acts as both

an initiator and promotor of carcinogenesis. It induces a variety of damage to DNA including the induction of cyclobutane thymine dimers, single strand breaks and DNA-protein crosslinking. The suppression of CH and DH responses induced following a single exposure to 10 kJ/m^2 of broadband UVB is reversed by immediate treatment of the irradiated site with liposomes containing T4N5, a dimer specific repair enzyme (Kripke *et al.*1992). Spleen cells taken from T4N5 treated, UVB irradiated mice, failed to transfer immunosuppression of CH responses. Evidence has been presented earlier that initiation of cutaneous carcinomas, particularly non-melanoma skin cancers, may involve UVB induced damage to the p53 gene. The data using T4N5 suggest that DNA damage also suppresses immune responses, with the possibility that DNA damage also acts as a promoter of tumourigenesis.

Using a monoclonal antibody to cyclobutyl thymine dimers, DC with DNA damage have been located in lymph nodes sections and in low-bouyant density cell populations following UVB irradiation (Sontag *et al.* 1995). However the numbers of DC displaying damage to their DNA was low. They peaked 24 hrs following irradiation, when 0.025% of cells in metrizamide enriched DC cell suspensions stained positively for thymine dimers. The percentage of DC showing DNA damage may be underestimated, expressed as it was as a percentage of total cells in a metrizamide cell suspension, which is not a pure DC population. Even if this so, the numbers of DC with DNA damage are still minute. The data showing actual numbers of DC accumulating in the lymph nodes following the irradiation of the mice, were not presented. However assuming that there are an average of 10000-15000 DC in DLN 24 hrs following exposure to a sub-erythemal dose of UVB, then the total numbers of DC with DNA damage would be between 0.25 and 7.5. Whether the low numbers of DC with damage is a result of rapid DNA repair or an accurate reflection of the numbers of LC-derived cells in the lymph node is uncertain. However, though these cells are found at low concentrations within the lymph node it is possible that they

contribute to the altered antigen presentation which has been proposed as a mechanisim of UVB-induced immunosuppression.

1.8.2.2 *Cis*-urocanic acid (UCA)

UV-B light exposure causes the isomerisation of *trans*-UCA, the naturally occuring isomer of UCA in the stratum corneum, to the more soluble *cis*-isomer. This is dose-dependent until the photostationary state is reached when about 50% of UCA is in the cis form (Norval *et al.*1989). *Cis*-UCA is found locally at high concentrations at the irradiated site, circulating systemically in the serum (Moodycliffe *et al.*1993) and is excreted in the urine. *Cis*-UCA was first proposed to be a mediator of UVB induced immunosuppression in 1983 (De Fabo and Noonan, 1983), because the action spectrum for UVB induced suppression of CH matched the absorbance spectrum for *trans*-UCA *in vitro*, both of which peaked at 270 nm. Further evidence for *cis*-UCA's involvement was gained using congenic mice which lack the gene that catalyses the formation of *trans*-UCA from histidine (De Fabo *et al.*1983). These mice had only 10% of the normal levels of UCA, and unlike the parent strain, the congenic mice were resistant to UVB-induced immunosuppression. However, this evidence has been followed by some conflicting data. Recently it has been found that the action spectrum for the isomerisation of *trans* to *cis*-UCA in mouse skin peaked at 310-315nm (Gibbs *et al.* 1993). In addition, *cis*-UCA is produced in the skin of mice following irradiation with lamps emitting in the UVB and UVA range (290-400). However shielded lamps emitting mainly in the UVA range (320-400nm), induced similar levels of *cis*-UCA as lamps emitting UVB, but failed to suppress CH responses (Reeve *et al.*1994).

Though the previous results suggest a complex relationship between *cis*-UCA production and immunosuppression, *cis*-UCA mimics some of the effects of UVB including depletion of LC from the epidermis (Kurimoto and Streilein, 1992; Moodycliffe *et al.*1993), suppression of CH responses via a TNF-α dependent mechanism (Kurimoto and Streilein, 1992) and the suppression of DH responses (Ross *et al.*1986). The production of a monoclonal antibody specific for *cis*-UCA has

provided clearer evidence for the action of *cis*-UCA. The reduction of LC numbers in the epidermis induced by UVB light (1440 J/m^2) or painting with *cis*-UCA (0.1mg in acetone painted onto the dorsum of each ear) (Moodycliffe *et al.*1993) was blocked by prior treatment with an anti-*cis*- UCA (El-Ghorr and Norval, 1995). In addition the anti-*cis*-UCA antibody blocked UVB-induced suppression of DH responses to HSV (El-Ghorr and Norval, 1995). However, unlike exposure to UVB, painting *cis* or *trans*-UCA (20 μg/ear) onto the ears does not induce accumulation of DC in draining lymph nodes (Moodycliffe *et al.*1992) and i.p injection of anti-*cis*-UCA did not block UVB induced DC accumulation (El-Ghorr and Norval, 1995).

In addition to the suppression of cutaneous DH and CH responses, there have been reports of cis-UCA suppressing allo-graft (Guymer and Mandel, 1993) and natural killer cell (Gilmour *et al.*1993) responses. Therefore though the mechanism by which *cis*-UCA exerts its immunosuppressive effects is complex, its ability to suppress a range of immune responses may make it a useful molecule for use therapeutically.

I.8.3 UVB-induced soluble suppressor factors

1.8.3.1 TNF-α

TNF-α is a pro-inflammatory cytokine which acts locally at sites of inflammation to activate endothelial cells to become adhesive for leukocytes. It induces phagocytic activity in a variety of cells, and cytokine production (IL-1, IL-6 and IL-8) from monocytes/macrophages. At higher concentrations it acts systemically on the hypothalamus and liver inducing fever and acute phase protein production respectively. High systemic concentrations of TNF-α, together with the other pro-inflammatory cytokines IL-1 and IL-6, are found during Gram-negative bacterial sepsis, and are associated with leukostasis, multi-organ failure and circulatory collapse. The TNF-receptor family (TNF-R) are glycoproteins with a single membrane spanning hydrophobic segment (Heller and Krönke, 1994). Recently there has been

evidence for the p55 TNF-R and the p75 receptor mediating different signals. It is interesting therefore, and may be of relevance to the effects of TNF-α reported in section 1.3.2, that there is epidermal/dermal specific TNF-R expression in human skin. The p55 TNF-R is expressed throughout the epidermis in normal human skin and on dendritic cells in the dermis, while the p75 TNF-R is expressed weakly on dermal DC and sweat ducts but there is no expression in the epidermis (Kristensen *et al.*1993).

TNF-α production is important in the skin immune system, being required for the induction (Cumberbatch and Kimber, 1995; Chavin *et al.*1991) and elicitation of contact sensitisation (Piguet *et al.*1991) in mice. TNF-α is produced in the skin in response to a wide variety of stimuli including; UVB exposure (Köck *et al.*1990) and a range of chemicals with sensitising, irritant and tolerogeneic properties (Enk and Katz, 1992b). TNF-α mRNA is detected 30 minutes after the application of hapten and peaks at 2-4 hrs (Enk and Katz, 1992b). In normal skin TNF-α activity is limited to the basal layers of the epidermis, while in involved psoriatic skin, activity is seen throughout the epidermis (Kristensen *et al.*1993). Keratinocytes are the main source of TNF-α in the epidermis (Oxholm *et al.*1991).

The role of TNF–α in UVB-induced immunosuppression is complex, as was described in section 1.3.2, it mediates LC migration from the epidermis and the accumulation of DC in the DLN (a requirement for the induction of immune responses), though it is also argued that it is involved in trapping LC in the epidermis. Although it is induced in the skin by treatments that result in the induction of immune responses and is vital for the induction of CH responses in mice in some systems (Piguet *et al.*1991), the local application of TNF-α to mice prior to sensitisation suppresses subsequent CH responses (Yoshikawa and Streilein, 1990). In addition neutralising antibodies to TNF-α can abrogate the effects of UVB-induced immunosuppression (Streilein, 1993; Moodycliffe *et al.*1994). The ability of TNF-α to

cause these disparate effects is possibly concentration dependent, with the resistance and susceptibility to UVB shown by different mice strains (section 1.7.5) being due to their differing responsiveness to the cytokine. There is some evidence for lower concentrations of TNF-α being active in suppressing CH responses in susceptible compared with resistant mice strains (Yoshikawa and Streilein, 1990).

In addition to causing the loss of LC from the skin, TNF-α may play a role in the recruitment of DETC into the skin. Investigators used clobetazole propionate to deplete DETC from the skin of C3H/HeN mice (Tamaki *et al.*1994). DETC-depleted skin was harvested and cultured with syngeneic epidermal cell suspensions for 3 days, during which time DETC migrated into the epidermis. DETC migration into the skin explants could be blocked using a neutralising antibody to TNF-α (Tamaki *et al.*1994). Although the role of DETC is not fully characterised, these cells express the γδ TCR which recognizes conserved epitopes such as heat shock proteins and therefore they may be involved in innate immune responses to infectious microorganisms. TNF-α, which is induced early in cutaneous inflammatory responses, may increase DETC migration into the skin, to provide an important innate immune mechanism to protect against disease. However, as discussed in section 1.1.2. there is evidence that DETC can alter the intensity of CH responses with the ratio of DETC to LC in the skin being the important variable. Therefore the ability of TNF-α to increase the numbers of DETC in the skin may detrimentally affect the ability of the skin to induce CH responses which may in part explain the immunosuppressive effects of TNF-α.

1.8.3.2 IL-10

There is now good evidence in mouse and human systems, that subsets of both CD4 and CD8 T cells exist which have differing functional activities. The most fully characterised of these is found in murine CD4 T cell clones which can be differentiated into 3 main subsets; Th0, Th1 and Th2, characterised by their ability to secrete certain patterns of cytokines. Though these cells have been easier to identify in mice, Th1 and Th2 subsets have also been described in humans (Romagnani, 1991). The Th0 subset represents an unrestricted phenotype which secretes IL-2, IFN-γ, GM-CSF, IL-3, IL-4, IL-5 and IL-10 (Mosmann and Moore, 1996). The two other subsets are identified by the cytokines that they secrete; Th1 cells secrete IL-2 and IFN-γ, Th2 cells secrete IL-4, IL-5, IL-6 and IL-10, while the cytokines IL-3, GM-CSF and TNF are secreted by both Th1 and Th2 clones (Mosmann and Moore, 1996). The development of Th1 and Th2 subsets seems to be dependent on the microenvironment that T cells are surrounded by during their activation, the concentration of IL-12 and IL-4 is particularly important, because IL-12 induces Th1 cells and IL-4 induces Th2 cells (Müller *et al.*1995). Once induced, the cytokines produced by Th1 cells such as IFN-γ , and IL-12 (produced by activated bystander macrophages) act to block cytokine synthesis by Th2 cells. Similarly Th2 effector cells inhibit Th1 cytokine synthesis by their production of IL-4 and IL-10. Hence the cytokines produced by each subset, act to increase the number of effector cells committed to their subset while acting as an antagonist to the induction of effector cells of the other subset. In a similar manner the effector responses induced by each subset will, due the cytokines that they secrete, inhibit the action of the other subset.

The functional activities of these clones are closely related to the cytokines they secrete. The secretion of IL-2 and IFN-γ, means that Th1 cells are potent activators of monocytes/macrophages and hence of cell-mediated inflammatory responses such as classical DH responses. Th2 cytokines on the other hand favour the production of humoral immune responses, with IL-4 being particularly important in class switching

to IgE. The description of Th1 and Th2 responses has provided a new framework for the control and fine-tuning of immune responses. Many of the concepts of Th1 and Th2 immunity fit with what is seen in cutaneous immune responses following UVB, with the suppression of cell-mediated responses and the alteration in humoral responses (Brown *et al.* 1995), suggestive of a shift towards a Th2 type of response. The cytokine IL-10 has been implicated with this shift to Th2-type immunity. The evidence for this will be discussed below.

IL-10 is a Th2-type cytokine, DNA coding for this cytokine has been isolated from mice and humans. Sequencing has shown a high level of conservation in the DNA coding for IL-10 in these species (Spits and de Waal Malefyt, 1992). IL-10 is produced by a wide variety of cells including B cells, cells of the monocyte /macrophage lineage, keratinocytes (Enk and Katz, 1992a; Grewe *et al.* 1995; Enk *et al.* 1995), Th2 clones (Mosmann and Moore, 1996), and, in humans, Th1 and cytotoxic T cell clones (Spits and de Waal Malefyt, 1992). It was first identified in 1989 as a cytokine synthesis inhibiting factor (CSIF) due to its ability to inhibit the production of IFN-γ by Th1 clones (Fiorentino *et al.* 1989). Since then, IL-10 has been shown to block most or all cytokine synthesis by Th1 and cytotoxic cell clones, by acting through an APC dependent mechanism (Fiorentino *et al.* 1991).

There are a number of studies which suggest that IL-10 contributes to the suppression of DH and CH responses which follows UVB exposure. Recombinant murine IL-10 (rIL-10) given i.p. suppressed the induction phase of DH but not CH responses (Schwartz *et al.* 1994). Thus IL-10 fails to mediate all the effects of UVB. However the results are interesting, infering that DH and CH responses may utilize different induction pathways. However, the effector phase of both DH and CH responses are suppressed by IL-10 (Schwartz *et al.* 1994), suggesting that their effector mechanisms are similar.

There seems to be good evidence that IL-10 acts as a down-regulatory signal in a wide variety of cutaneous immune responses. Certainly it is produced by keratinocytes, the main source of IL-10 in skin, not just following UVB (Enk *et al.* 1995) but also in response to contact sensitisers (Enk and Katz, 1992a).Therefore IL-10 production in itself is not associated with the suppression of cell-mediated immune responses which follows UVB. Instead, it is likely to provide an anti-inflammatory check to reduce damaging inflammation. Indeed mice which lack IL-10 show unrestrained irritant responses which in some cases results in irreparable tissue damage (Berg *et al.* 1995). This anti-inflammatory activity of IL-10 may reflect its ability to reduce pro-inflammatory and chemotactic cytokine production by macrophages (Spits and de Waal Malefyt, 1992).

In order to understand the different effects of IL-10, induced by antigen challenge and by UVB, it is necessary to consider the kinetics of IL-10 production. It is produced late in the normal inflammatory response following sensitisation, with no transcription of IL-10 mRNA within the first 4 hrs and peak production at 12 hrs (Enk and Katz, 1992a). Thus in primary responses, keratinocyte-derived cytokines such as TNF-α and GM-CSF will have been up-regulated, LC will have processed antigen, received their signal to migrate and be initiating contacts with T cells in the DLN before IL-10 is induced. Similarly in secondary responses the presence of IL-10 between 4-24 hrs following challenge will correspond with the inflammatory response on its upward sweep or at its peak and provide a beneficial anti-inflammatory effect.

This contrasts with what is seen following UVB exposure, IL-10 mRNA transcription is induced in keratinocytes 8 hrs after irradiation (Enk *et al.* 1995). When an irradiated site is sensitised, the presence of UVB-induced IL-10 results in LC processing antigen in a cutaneous environment rich in IL-10. This alteration in the epidermal microenvironment may affect the activity of LC as APC. Certainly it has been reported that IL-10 reduces the ability of DC to induce IFN-γ production from

CD4 and CD8 T cells in a primary MLR *in vitro* (Macatonia *et al.*1993). The role of UVB-induced IL-10 in the secondary response seems clearer. The pre-emptive production of IL-10 prior to challenge in the secondary response means that the anti-inflammatory signal is present before the inflammation process begins and hence causes the suppression of the normal inflammatory response.

1.9 The aims of this investigation

1.9.1 Chronic UVB exposure

Chronic UV exposure protocols have been used to look at skin ageing and UV-induced cancers, but little work is available on the effects of chronic UVB on the SIS. Therefore, the aim of this study was to examine the effect of chronic broad- and narrowband UVB on LC numbers, SBC numbers and *cis*-UCA isomerisation at exposed skin sites. In addition the ability of chronically irradiated skin to support the induction and elicitation of CH responses will be studied.

1.9.2 The effect of acute UVB-exposure on DC function

Acute low-dose UVB-exposure causes the suppression of a variety of immune responses (section 1.7), by mechanisms involving multiple mediators (section 1.8). *In vitro* studies have suggested a role for UVB-induced alterations in LC function in the induction of immunosuppression. These studies demonstrated that the exposure of freshly isolated epidermal LC to low-dose UVB *in vitro* reduced the subsequent up-regulation of their accessory activity during culture (Tang and Udey, 1991; Rattis *et al.*1995). However the effect of *in vivo* UVB exposure on the function of LC-derived DC, has not been addressed directly. Therefore in this study an *in vivo* model has been used in an attempt to determine the effect of acute UVB-exposure on the accessory activity of DC in lymph nodes draining UVB-exposed skin.

1.9.3 The effect of acute UVB exposure on DC phenotype

In addition to altering the function of LC, *in vitro* UVB exposure also alters their phenotype. The exposure of freshly isolated murine LC to low-doses of UVB *in vitro*

inhibits the up-regulation of ICAM-1 expression (Tang and Udey, 1991), B7-1 and B7-2 expression (Weiss *et al.* 1995) which occurs during culture. However no studies have addressed whether UVB exposure affects the phenotype of LC-derived DC *in vivo*. Therefore the aim of this study was to examine the phenotype of DC in skin-DLN following acute low-dose UVB-exposure.

1.9.4 Strain differences in IL-6 production

The application of sensitising chemicals to the skin of mice results in the induction of proliferative responses in the DLN (Scholes *et al.*1992). The lymph node proliferative response is associated with the production of IL-6 (Hope et al. 1994), the main source of which appears to be DC (Hope *et al.*1995; Cumberbatch *et al.* 1996). Therefore it is possible that the production of IL-6 by lymph node DC plays a role in the initiation of the T cell proliferation, and their differentiation into effector cells. Because of this it was decided to examine the effect of UVB-exposure on IL-6 production in lymph nodes and also to address whether strain differences in the production of this cytokine exist in UVB susceptible and UVB resistant strains of mice.

Chapter 2. Materials and methods

2.1. General

2.1.1 Mice

Female C3H/HeN, BALB/c, C57BLK/6 strain mice aged 6-8 weeks, were obtained from the specific pathogen-free animal breeding facility at the Medical Microbiology Transgenic Unit, University of Edinburgh. Mice of DBA/2 and AKR strains were purchased (B&K Universal Ltd, Hull, UK) at 3 weeks of age and housed in the Transgenic unit until they were ready for use.

2.1.2 Contact sensitisers

Fluorescein isothiocyanate (FITC) and 4-ethoxymethylene-2-phenyloxazol-5-one (oxazalone) were obtained from Sigma-Aldrich Co. (Poole, UK). FITC was dissolved in 1:1 acetone:dibutylphthalate (DBT) at 5%, 2.5% and 1% w/v and oxazolone was made up in the same vehicle at 1% and 0.25% w/v. In some experiments sensitisers were dissolved in 4:1 acetone:olive oil (AOO).

2.1.3 Growth media

2.1.3.1 Standard RPMI-FCS

RPMI-1640 medium (Flow Laboratories Ltd., Irvine, Ayrshire) was supplemented with 100 i.u./ml penicillin, 200μg/ml streptomycin, 2mM L-glutamine, 100μg/ml gentamicin and 10% heat-inactivated foetal calf serum (Gibco BRL, Paisley, UK).

2.1.3.2 RPMI-HEPES

RPMI-FCS was supplemented with 25mM HEPES.

2.1.3.4 UVB sources and exposure

Mice were irradiated on the ears or shaved backs under two Philips TL-20W/12 bulbs with an output range of 270-350nm, peak 305nm, emitting 80mW/ cm^2. The output of this source was determined by Dr. Neil Gibbs (Photobiology Unit, University of Dundee) using a filtered photodiode meter, calibrated against measurements made with a UV-visible spectroradiometer (model 742, Optronic Laboratories Inc.) across the spectral range 250-400nm. The tube to target distance was 16 cm and mice were contained in separate compartments in a perspex box to prevent shielding by littermates. One minimal erythema dose (MED) for C3H/HeN mice under these conditions was 1500 J/m^2.

In the chronic UVB exposure studies a single Philips TL01 lamp was used as a source of narrow-band UVB (Van Weelden et al. 1988). This lamp emitted predominantly at 311-312nm, and the total irradiance was 200 $\mu W/cm^2$ (1 watt=1J/sec). One MED for C3H/HeN mice was 10080 J/m^2 of TL01.

2.2. Chronic UVB exposure study

2.2.1 UVB exposure

Mice were exposed on their shaved backs to 500 or 1000 J/m^2 of broadband UVB (TL12) or 3000 J/m^2 narrow-band UVB (TL01), 3 times a week (Monday, Wednesday and Friday) for up to 6 weeks. The backs of mice were shaved on the Monday of each week, prior to their first UVB exposure. Their ears were not protected from irradiation.

2.2.2 Staining epidermal sheets for ATPase activity

Three days after the last irradiation, the ears of four mice were removed and split into dorsal and ventral halves. Epidermal sheets were prepared from the dorsal halves by floating them epidermal side down in 0.76% tetrasodium ethylenediamine tetraacetic acid (EDTA) in phosphate buffered saline (PBS), pH 7.2 for 2 hrs at 37°C. Epidermal sheets were then removed carefully with a scalpel blade and stained for adenine triphosphatase

(ATPase) using adenine diphosphate (ADP) as a substrate (Chaker *et al.*1984). Briefly, the epidermal sheets were fixed in sodium cacodylate/formaldehyde solution (6.85% sucrose, 1.6% sodium cacodylate and 0.1% formaldehyde in distilled water) at 4°C for 1 hour. The sheets were rinsed 3 times in PBS and incubated with ADP-lead at 37°C for 70 minutes. The sheets were rinsed 3 times and incubated in ammonium polysulphide for 20 minutes at room temperature. The epidermal sheets were finally rinsed in tap water, and mounted in 50% glycerol under a glass cover slip. The specimens were coded and examined 'blind' under the microscope. The number of ATPase+ cells were counted in at least 40 fields per group (1 field=0.1mm^2)

2.2.3 Quantitation of sunburn cell (SBC) density and epidermal thickness

Single ears from each of three mice per group were removed 3 days following the last irradiation and were fixed in 10% formyl saline before routine histological processing. To avoid duplicate sectioning of the same SBC only every fifth 8μm section was taken and stained with haematoxylin and eosin. The stained sections were examined on a light microscope attached to a See-Scan Image analyser. This computer was used to calculate the number of SBC per cm of interfollicular epidermis. Twelve sections were scored for SBC for each group. This represented between 30-50 cm of interfollicular epidermis. For each section, the number of SBC/cm was calculated and the data expressed as the mean number of SBC/cm± standard error mean (SEM). The SBC were characterised as having a pyknotic nucleus surrounded by an eosinophilic and/or vacuolated cytoplasm. In addition the thickness of the epidermis was measured by the computer at approximately 100 sites per group.

2.2.4 Analysis of urocanic acid isomers

Single ears from each of three mice in each group were collected 3 days after the last irradiation, weighed and their concentration of *cis* and *trans*-UCA analysed by HPLC by Dr. John Crosby (Department of Chemistry, University of Bristol). The mean amount of each isomer measured in mg/g wet weight of ear and the percentage *cis*-UCA was calculated for each group.

2.2.5 Measurement of contact hypersensitivity

The contact hypersensitivity (CH) reaction to oxazolone was measured 5 hrs after the last irradiation. Briefly, 9 mice per group received a sensitising dose of 50µl 1% oxazolone in vehicle (AOO) on their shaved backs. A negative control group received 50µl of the vehicle (AOO) alone. Six days later the ears of each mouse were measured using an engineers' micrometer (Draper SM-510, Japan) and challenged with 25µl 0.25% oxazolone in vehicle. Twenty four hrs later the thickness of each ear was remeasured, the increase in ear thickness was calculated, and the results expressed as the mean challenge-induced increase of ear thickness in $mm^{-2} \pm$ SEM.

2.3. Dendritic cells and UVB-induced immunosuppression

2.3.1 Contact hypersensitivity responses

To measure the effect of acute UVB-exposure on CH responses, the shaved backs of C3H/HeN and BALB/c mice were exposed to two doses of 1440 J/m^2 of UVB, 48 and 24 hrs prior to sensitisation. In initial experiments mice were exposed to UVB without ear protection. However, the ears of BALB/c mice showed UVB-induced damage, so in subsequent experiments the ears were protected. To do this, the mice were anaesthetised by interperitoneal injection of 0.1 ml sterile distilled water containing 0.83 mg/ml hypnovel (Roche, Welwyn Garden City, UK) and 1.67 mg/ml hypnorm (Janssen Pharmaceutical, Oxford, UK). One group (n=7) were irradiated in a perspex box, and

their heads were covered with aluminium foil to protect the ears from UVB exposure. The other two groups (n=7) were anaesthetised but were not irradiated. Twenty four hrs later the irradiated group and one of the unirradiated control groups were sensitised on the back with 50µl 1% oxazolone, the backs of the third group were painted with 50µl vehicle (AOO).

Schematic of CH protocol

		Day -2, -1	Day 0	Day 6
	anaesthetised	**UVB**	**1% Ox**	**0.25% Ox**
Group 1	+	+	+	+
Group 2	+	—	+	+
Group 3	+	—	—	+

Six days later the ears of each mouse were measured (see section 2.2.5) and challenged with 25µl 0.25% oxazolone in vehicle. Twenty four hrs later the ears of each mouse were remeasured, the increase in ear thickness was calculated, and the results expressed as the mean challenge-induced increase of ear thickness in $mm^{-2} \pm$ SEM. Percentage suppression of CH= 100—[(a—b)/(c—b)x100], where a=the mean increase in ear thickness from Group 1 (UVB) above, b=the mean increase in ear thickness from group 3 (—ve) and c=the mean increase in ear thickness from Group 2 (+ve).

2.3.2 UVB-induced changes in DC function and phenotype

2.3.2.1 Enrichment of DC

The method of Macatonia *et al.* 1986 was followed in outline to prepare enriched DC populations from pooled auricular or peripheral lymph nodes following a variety of treatments, see sections 2.3.2.2, 2.3.5.2. A single cell suspension of LNC was prepared by mechanical disaggregation through 200-mesh stainless steel gauze (J. Stanier and Co. Manchester, UK.). Cells were washed and viable cells counted by exclusion of 0.5% trypan blue. Cells were resuspended in standard RPMI-FCS. DC-enriched populations

were prepared by density gradient centrifugation on Metrizamide (Nygaard, Oslo, Norway). Briefly LNC were adjusted to $5x10^6$ cells/ml in RPMI-FCS and 8ml of the cell suspension was gently underlayed with 2ml of 14.5% Metrizamide in RPMI-FCS and centrifuged for 20 minutes (600g) at room temperature. The low buoyant density population (DC^+) that accumulated at the interface was collected and washed twice with RPMI-FCS. This population comprised 50-70% DC as judged by morphology using light microscopy. Analysis of these enriched DC populations by flow cytometry (see section 2.3.4) revealed a corresponding population of large granular cells with high levels of membrane Ia expression. In some experiments the pellet, which comprised LNC depleted of DC (DC^-), was collected and washed in RPMI-FCS. This population contained around 2% of cells with DC morphology. Flow cytometric analysis confirmed that around 2% of cells in this population had the characteristics of DC- large granular cells with high Ia expression.

2.3.2.2 DC as antigen specific accessory cells

The method described by Jones et al 1989 was used. The ears of C3H/HeN mice (n=3-5) were painted with 25µl of FITC or oxazolone. Seven days later mice were killed, and LNC prepared from the auricular lymph nodes as described above and used as responder populations. These sensitised LNC were resuspended at $5x10^6$ cells/ml in RPMI-FCS and 100µl of cells added per well in a round bottomed 96-well microplate (Nunclon, Roskilde, Denmark). DC were enriched as described (section 2.3.2.1) from the auricular lymph nodes of C3H/HeN mice sensitised with FITC or oxazolone 18 hrs previously. In some experiments the ears of mice were irradiated with 1440 J/m^2 of UVB 48 and 24 hrs prior to sensitisation. These enriched DC^+ populations were added to wells to provide DC:responder ratios of either 1:20, 1:40, 1:55 or 1:100. Plates were cultured for 48 hrs in a humidified atmosphere of 5% CO_2 in air, and, 18 hrs prior to culture termination, 0.7µCi ^{3}H-methyl thymidine, specific activity 84Ci/mmol (Amersham Life Science, Little

Chalfont, UK) were added to all wells. Cultures were terminated by automatic harvesting and ^{3}H-methyl thymidine incorporation measured in counts per minute (CPM) using a liquid scintillation analyser (Canberra Packard, Zurich, Switzerland). The data are presented as the mean CPM±SD from 5 replicate cultures. Results are expressed as the mean counts per minute (CPM)± standard deviation (SD) from 5 replicate cultures.

2.3.3 Identification of FITC-bearing cells in the draining lymph node

DC-enriched and depleted populations were prepared (see section 2.3.2.1) from the DLN of mice sensitised with 2.5% FITC or 1% oxazolone 18 hrs previously. A minimum of 10^4 cells from each population was analysed using a Coulter XL flow cytometer. Non-viable cells were gated out, and background controls were set at 1% using DC-enriched and depleted populations isolated from the DLN of oxazolone-sensitised mice. DC were identified by forward and side angle light scatter characteristics. The percentage of DC bearing FITC and the intensity of FITC staining were recorded.

2.3.4 Phenotypic analyses

DC were enriched, as described above, from the auricular or pooled peripheral lymph nodes of naive C3H/HeN mice, or from the auricular lymph nodes following irradiation with the broadband UVB source (TL-12) 48 and 24 hrs previously. In some experiments DC were enriched from the auricular lymph nodes 18-24 hrs after sensitisation with oxazolone (n=10-15). The effect of UVB on this system was examined by exposing mice to UVB 48 and 24 hrs before sensitisation. A minimum of 10^5 DC was incubated on ice for 30 minutes with the appropriate isotype control (anti-human MHC class I or anti-dinitrophenyl (DNP)), or the primary monoclonals listed in Table 2.1. Monoclonal antibodies recognising Ia (Serotec, Oxford, UK) and B7-2 (CD86) (Pharmingen, San Diego, USA) were purchased, and the ICAM-1 monoclonal was acquired as a hybridoma

Specificity	Species/Isotype	Clone	Supplier
human MHC class I	Rat/IgG2b	YTH862.2	Serotec
DNP hapten	Rat/IgG2a	LO-DNP-16	Serotec
murine ICAM-1	Rat/IgG2b	YN-11.7.4	(Takei, 1985)
murine Ia	Rat/IgG2a	YE2/36HLK	Serotec
murine B7-2	Rat/IgG2a	GL1	Pharmingen

Table 2.1 Monoclonal antibodies used in this study.

culture supernatant (Takei, 1985). The cells were washed once with 2ml of RPMI-FCS and incubated with an affinity purified $F(ab')_2$ rabbit anti rat IgG-FITC conjugate (Serotec, Oxford, UK) for 30 minutes on ice. Finally, the cells were washed once more in RPMI-FCS, fixed in 1% formyl saline and analysed using a Coulter XL flow cytometer.

Cells were identified first using their forward scatter (FS) and side angle light scatter (SS) characteristics to quantify their size and granularity. Regions were placed around cells with low FS and SS (contaminating cell population), cells with high FS and SS (putative DC) and around the total cell population. The events within each region were then displayed on histograms of log fluorescence intensity (x-axis) against cell count (y-axis). A minimum of 5000 events in the DC region was accumulated. Isotype controls were routinely set at 1%. The percentage of DC expressing each marker and the density of expression (mean fluorescence intensity) of the marker were recorded.

In some experiments enriched DC populations were resuspended to around 10^5 cells/ml and cytospins of 10-15^4 cells prepared. Cytospin slides were air dried and fixed in acetone. They were then stained for ICAM-1 and MHC class II expression using peroxidase conjugated avidin staining with a Sequenza (Shandon, Life Science International, Basingstoke, UK). The Sequenza is a semi-automated apparatus for histochemical staining, slides are placed in disposable holders which allow a controlled flow of solution over the sample. The slides were first rinsed with distilled water and then with Tris buffered saline (TBS) for 5 minutes. The slides were then blocked for 20 minutes with normal rabbit serum diluted 1:5 in TBS. Next the slides were incubated for 30 minutes with 100µl of the monoclonal antibodies against Ia or ICAM-1 or an isotype control antibody (sources described above) diluted optimally. Each slide was then incubated for 30 minutes with 100µl of biotinylated rabbit-anti-mouse immunoglobulin (Vector Laboratories, Peterborough, UK) diluted to 1:400 in TBS. Next every slide was incubated for 30 minutes with horseradish peroxidase labelled avidin (Vector

Laboratories) at 1:1000 in TBS. The slides were washed with TBS, and incubated with the peroxidase substrate (1x10 mg 3,3'-diaminobenzidine tetrahydrochloride tablet (Sigma, St Louis, USA) in 10ml of distilled water with 14µl of hydrogen peroxide added). Finally the slides were rinsed in distilled water, counterstained with haematoxylin and mounted under a coverslip. Using this technique positive staining appeared brown.

2.3.5 The effect of UVB on mixed lymphocyte reactions

2.3.5.1 Preparation of LNC

In initial experiments LNC were isolated from the pooled peripheral lymph nodes of BALB/c mice. Later, responder LNC were prepared from pooled auricular lymph nodes isolated from BALB/c strain mice (n=3-5) which had been painted 48 hrs earlier with 1% oxazolone on the dorsum of both ears. LNC were prepared as described above (section 2.3.2.1) and cultured at $1.5x10^5$ cells/ml in RPMI-FCS, unless otherwise stated in the text.

2.4.5.2 Mixed lymphocyte reaction

C3H/HeN DC^+ populations were prepared from the auricular lymph nodes of unsensitised mice (n=10-15), mice exposed to low dose (1440 J/m^2, -48, -24 hrs) or to high dose (15 kJ/m^2, -24 hrs) UVB treatment or another stated protocol. Control DC^+ populations were enriched from the skin draining lymph nodes of unirradiated and unsensitised mice (n=10). Cells with DC morphology were counted and a known number of C3H/HeN DC was cultured with BALB/c responder LNC at various stimulator: responder ratios. DC^- populations were also used as stimulators. These cells were cultured alone or with BALB/c LNC at matching stimulator:responder ratios as with the DC^+. Plates were cultured for 120 hrs in a humidified atmosphere of 5% $C0_2$ in air. Cells were treated for the final 18 hrs of culture with 0.7 µCi ^{3}H-methyl thymidine. The cells were harvested and thymidine incorporation was measured as described in section 2.3.2.2. Results were expressed as mean CPM±SD from 5 replicate cultures.

2.4. Analysis of cytokine production during the induction phase of contact sensitisation.

2.4.1 Cytokine production and proliferation of draining lymph node cells

Groups of mice (n=5) received either 25µl of oxazolone, or an equal volume of vehicle (AOO) alone (n=10), on the dorsum of both ears. At various times thereafter (2, 3 and 5 days later), mice were killed and their auricular lymph nodes excised and pooled for each experimental group. Single cell suspensions of LNC were prepared by mechanical dissaggregation through 200 mesh stainless steel gauze, cells were then washed and resuspended in RPMI-FCS. Viable cell counts were performed by exclusion of 0.5% trypan blue. Cells were resuspended at $1x10^7$ LNC per ml in RPMI-HEPES and seeded into separate plates for measurement of cytokine production and LNC proliferation. To analyse cytokine production, 1ml of cell suspension from each group was cultured for 24 or 48 hrs in 24 well tissue culture plates (Costar, Cambridge, Ma.). Culture was terminated by centrifugation and the supernatants were stored at -70°C until analysis. The concentration of IL-6 and IFN-γ in the supernatant was analysed by ELISA (section 2.4.5 and 2.4.6 respectively), and results expressed as nanograms of cytokine per ml (ng/ml). To measure proliferation, 5 aliquots of 200µl of LNC at $1x10^7$ cells/ml were seeded into 96 well round bottomed tissue culture plates (Nunc, Roskilde, Denmark). Cells were cultured for 24 hrs with 1µCi/well ^{3}H-methyl thymidine. The cultures were terminated by automatic harvesting and ^{3}H-methyl thymidine incorporation measured (see section 2.3.2.2). The data are presented as the mean CPM±SD from 5 replicate cultures.

2.4.2 Intracellular IL-6 in draining LNC

Mice received either 25µl of oxazolone (n=10-15) or an equal volume of vehicle control (n=10-15) on the dorsum of both ears. Three days later groups of mice were killed and their auricular lymph nodes excised and pooled. Single cell suspensions were prepared as above and cells were resuspended at $2x10^8$ LNC in 1ml of RPMI-HEPES. The cell suspensions were put on ice and sonicated for 3x10 second cycles (Sanyo Soniprep 150, Japan). Finally, the suspensions were microfuged at $1.5x10^4$g for 5min, the supernatant was collected and stored at -70°C until analysis for IL-6 by ELISA (see section 2.4.5). In some experiments LNC from oxazalone sensitised mice were cultured for 24 hrs at $1x10^7$ cells/ml. Following culture the cells were washed once, resuspended at $2x10^8$ cells/ml and sonicated as above.

2.4.3 The effect of UVB on LNC proliferation, IL-6 production and intracellular IL-6 levels

Groups of mice (n=5) were exposed to a single dose of 1440 J/m^2 UVB using the TL-12 source described earlier, or were not irradiated (n=3). One, three and five days later the auricular lymph nodes from irradiated animals and the peripheral lymph nodes from unirradiated animals were collected and pooled for each group. To examine IL-6 production, a single cell suspension was prepared and separate cultures were prepared for supernatants and proliferation as described above (section 2.4.1). Intracellular levels of IL-6 were examined by sonicating LNC suspensions (see section 2.4.2). The supernatants were collected and stored at -70°C until analysis for IL-6 by ELISA (section 2.5.5).

2.4.4 Kinetics of cytokine production in the skin

The backs of mice were shaved 24 hrs prior to sensitisation and any mice with lesions were discarded. Mice were sensitised with 100µl of oxazolone, the same volume of vehicle (AOO) or were left unpainted. Groups of mice (n=4) were killed 0, 1, 2, 4, 8 or 24 hrs later, and samples of skin weighing between 0.04 and 0.1g were collected from the treated site. Each sample was chopped finely, weighed and resuspended in 1ml of RPMI-FCS medium in a 1.5ml eppendorf cuvette (Alpha Laboratories, Eastleigh, UK). Each cuvette was snap frozen in liquid nitrogen, thawed and the contents were homogenised with a tissue grinder. The homogenised sample was snap frozen again, thawed and the contents sonicated for 10 seconds (Soniprep 150, Sanyo). Debris was removed by centrifugation (5min at $1.5x10^4$g) and the supernatant was assayed for IL-6 by ELISA (section 2.4.5) and the results expressed as nanograms of cytokine per gram of original tissue (ng/g).

2.4.5 Measurement of IL-6 using a cytokine-specific sandwich enzyme-linked immunosorbent assay (ELISA)

IL-6 was analysed by a cytokine-specific sandwich ELISA, developed originally by Dr. R. J. Dearman, Zeneca Central Toxicology Laboratory, Macclesfield (Dearman *et al.* 1993). Flat-bottomed 96-well maxisorb plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 50µl/well of rat anti-murine-IL-6 at 2.5 µg/ml (Genzyme, West Malling, UK) diluted in 0.1 M carbonate buffer (pH 9.6). The plates were given 3x3minute washes using PBS with 0.05% w/v Tween 20. This process will be referred to as washing throughout this section. Plates were then blocked with 100µl/well of 10% FCS in PBS and incubated at 37°C for 30 minutes. For each experiment doubling dilutions of recombinant murine-IL-6 (rIL-6), specific activity $10x10^8$ units/mg (Genzyme) covering a concentration range of 78-20000pg/ml were plated out in triplicate (100µl/well) in RPMI-FCS which was used for all the ELISA experiments. Sample supernatants were also plated out in triplicate (100µl/well) and the control wells received RPMI-FCS. All the plates were incubated at room temperature (RT) for 2hrs. After

washing, 100µl/well of goat anti-murine-IL-6 (R&D Systems, Abingdon, UK) at 8µg/ml diluted in RPMI-FCS were added to each well and the plates incubated at RT for 2hrs. The plates were washed and 100µl/well of 1:500 dilution of donkey anti-goat IgG/horseradish peroxidase conjugate (Serotec, Oxford, UK) in RPMI-FCS were added to all wells and plates incubated at RT for 2hrs. The plates were washed and 100µl/well of enzyme substrate (*o*-phenylenediamine dihydrochloride and urea hydrogen peroxide) was added. The substrate was added to all wells except column 1 which received 100µl/well of 0.5 M citric acid. After 15 minutes incubation in the dark, 50µl/well of citric acid was added to all wells to stop development of colour. The optical density (OD) of each sample was measured using a plate reader at 450nm and a standard curve plotted using the OD_{450nm} against the concentration of IL-6 in ng/ml on a log scale. The standard curve was used to calculate the concentration of IL-6 in ng/ml ± standard deviation (SD) in the triplicate samples. The limit of detection of IL-6 was 0.15ng/ml.

2.4.6 Measurement of IFN-γ using ELISA

IFN-γ was analysed by cytokine-specific sandwich ELISA, developed originally by Dr. R. J. Dearman, Zeneca Central Toxicology Laboratory, Macclesfield (Hodgson *et al.* 1996). Flat-bottomed 96-well maxisorb plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100µl/well of rat anti-murine-IFN-γ at 0.5µg/ml (Genzyme) diluted in 0.1 M carbonate coating buffer (pH 9.6). The plates were washed using PBS with 0.05% w/v Tween 20. Plates were then blocked with 100µl/well of 5% FCS in PBS and incubated at 37°C for 30 minutes. For each experiment doubling dilutions of recombinant murine-IFN-γ (rIFN-γ), specific activity 0.33×10^8 units/mg (Genzyme) covering a concentration range of 39-10000pg/ml were plated out in triplicate (100µl/well) in RPMI-FCS. Sample supernatants were also plated out in triplicate (100µl/well) and the control wells received RPMI-FCS. All the plates were incubated at room temperature (RT) for 2hrs. After washing, 100µl/well of goat anti-murine-IFN-γ (Genzyme) diluted in RPMI-FCS were added to each well and the plates incubated at RT for 2hrs. The

plates were washed and 100µl/well of 1:500 dilution of donkey anti-goat IgG/horseradish peroxidase conjugate (Serotec, Oxford, UK) in RPMI-FCS was added to all wells and plates incubated at RT for 2hrs. The plates were washed and 100µl/well of substrate (as above) 100µl/well of citric acid. After 15 minutes incubation in the dark, 50µl/well of citric acid was added to all wells to stop development of colour. The optical density (OD) of each sample was measured using a plate reader at 450nm and a standard curve plotted using the OD_{450nm} against the concentration of IFN-γ in ng/ml on a log scale. The standard curve was used to calculate the concentration of IFN-γ in ng/ml ± standard deviation (SD) in the triplicate samples. The limit of detection of IFN-γ was 0.15ng/ml.

2.5 Statistics

The non-parametric Mann-Whitney test was used for the analysis of differences between groups. Mean differences were considered statistically significant if $p<0.05$.

Chapter 3. Results

3.0. The effect of chronic exposure to UVB on the skin and cutaneous immune responses

3.0.1 Introduction

The effect of UVB on the immune system of mice has been studied mainly following acute exposure, either after a single dose of UVB, or up to 4 consecutive daily exposures. The immunosuppressive effects of acute UVB exposure have been discussed in section 1.7 and include the inhibition of responses to infectious agents, tumours and contact sensitisers. UVB alters the cutaneous microenvironment, causing the loss of LC, specialised epidermal APC, from the skin. It also isomerises *trans*-UCA, present in the stratum corneum, to the more soluble *cis*-isomer. Both of these changes have been proposed as mediators of UVB-induced immunosuppression (section 1.8).

A narrowband UV lamp (TL01) emitting at 311-312 nm has been developed for use in phototherapy (Van Weelden *et al.*1988) This source causes less skin damage, measured as a function of erythema and sunburn cell (SBC) induction, but efficiently converts *trans*-UCA to *cis*-UCA (Gibbs *et al.*1993). In addition, acute TL01 exposure has been reported to reduce the density, but not the antigen presenting function, of epidermal LC (El-Ghorr *et al.*1994). The output of the broad- and narrowband sources are shown in Figure 3.0.1.

The effects of chronic suberythemal UVB have received little attention and there have been no attempts to examinine the effects of chronic narrowband irradiation. However, since most individuals are exposed to UVB in a chronic low-dose form in their everyday lives, the effects of chronic UVB on the skin and the SIS are of importance. It was decided therefore to compare the effect of chronic low-dose broadband UVB and narrowband TL01 exposure on the number of LC and SBC in the epidermis, the epidermal thickness and *cis*-UCA conversion. The ability of mice to generate CH

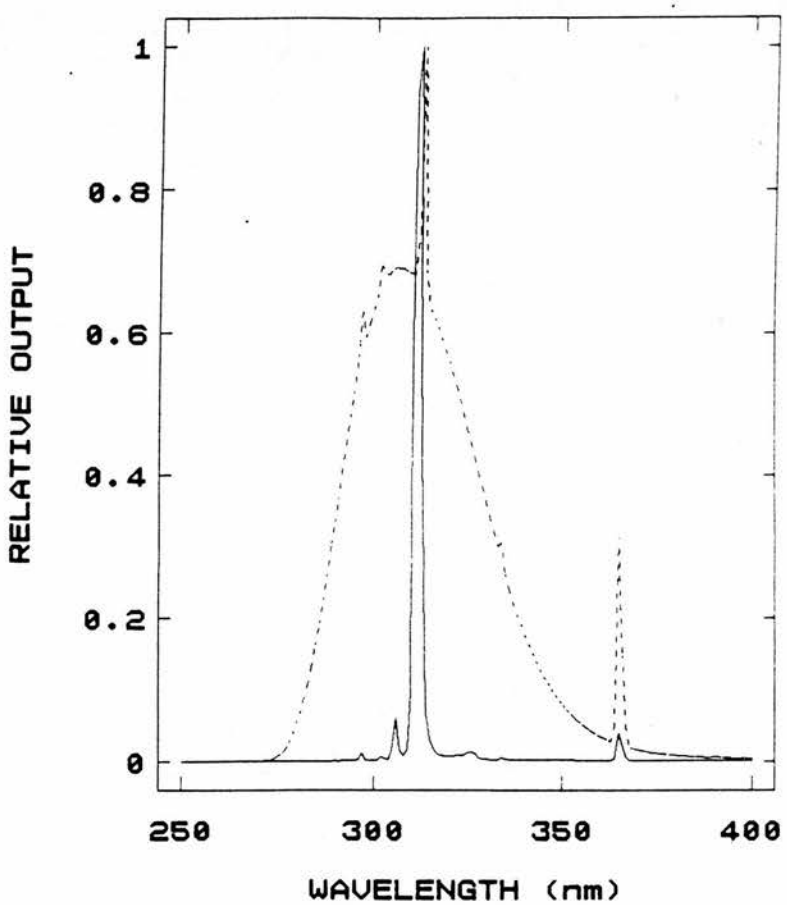

Figure 3.0.1 The emission spectra for the broadband UVB lamp (TL12 dotted line) and the narrowband TL01 lamp (solid line) as in Figure 1.2 in the introduction. Reproduced from Moodycliffe *et al.* 1994.

responses following six weeks of irradiation was also investigated. To do this, the shaved backs of mice were exposed to 500 or 1000 J/m^2 of broadband UVB (TL12 source) or to 3000 J/m^2 of narrowband UVB (TL01 source) three times a week (Monday, Wednesday and Friday) for up to 6 weeks. The MED for C3H/HeN was 1500 J/m^2 of broadband UVB (TL12) and 10080 J/m^2 of narrowband UVB (TL01). At various timepoints skin samples were taken for analysis and after 6 weeks CH responses were examined.

3.0.2 The effect of broadband and narrowband UV on the number of ATPase$^+$ cells

In the epidermis only LC express the ATPase enzyme on their cell surface. Ultraviolet-B irradiation with 500 or 1000 J/m^2 three times weekly resulted in approximately 50% and 70% reduction in the density of ATPase$^+$ cells, respectively, after 2-3 weeks (Figure 3.0.2). This reduction was statistically significant ($p \leq 0.01$) and maintained throughout the period of UVB irradiation although there was a tendency for LC numbers to begin to return to background levels as the number of exposures continued. Morphologically, between 20-40% of LC appeared to lose their dendrites following 1-4 weeks of UVB exposure. After 5-6 weeks, however, this number was 7-10%. Exposure to TL01 resulted in a steady decline in the density of LC at the three time points tested (Figure 3.0.2) but there was no effect on dendritic morphology.

3.0.3 The effect of the two UVB sources on SBC and epidermal cell thickness

One indication of photodamage is the presence of SBC in the epidermis (Young, 1986) and are thought to represent apoptotic cells (Schwartz *et al.* 1995). Figure 3.0.3 shows a SBC in the epidermis following 2 weeks of broadband UVB (1000 J/m^2) After 1 week of exposure to UVB (500 J/m2 or 1000 J/m^2) SBC had started to appear and their numbers increased with further exposure in a dose-dependent manner (Figure 3.0.4). One week of 3000 J/m^2 of TL01 exposure, however, induced few SBC. After 2 weeks of TL01, SBC did appear but their numbers remained lower than those

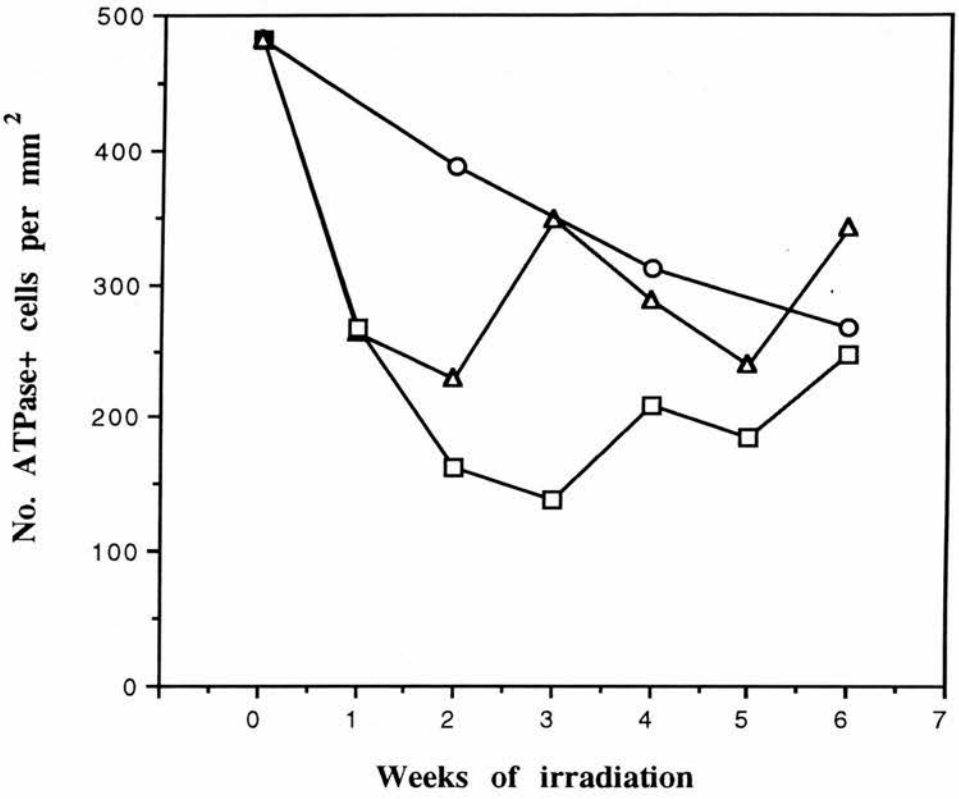

Figure 3.0.2 The effect of chronic UVB on the number of LC in murine epidermis. Ears were removed 3 days after the last irradiation and epidermal sheets stained for ATPase activity. Triangles represent 500 J/m^2 UVB, squares 1000 J/m^2 UVB and circles 3000 J/m^2 TL01 exposure. The standard error of the mean at each time point was <25. All values were significantly lower ($p<0.05$) than non-irradiated age-matched controls (time point 0).

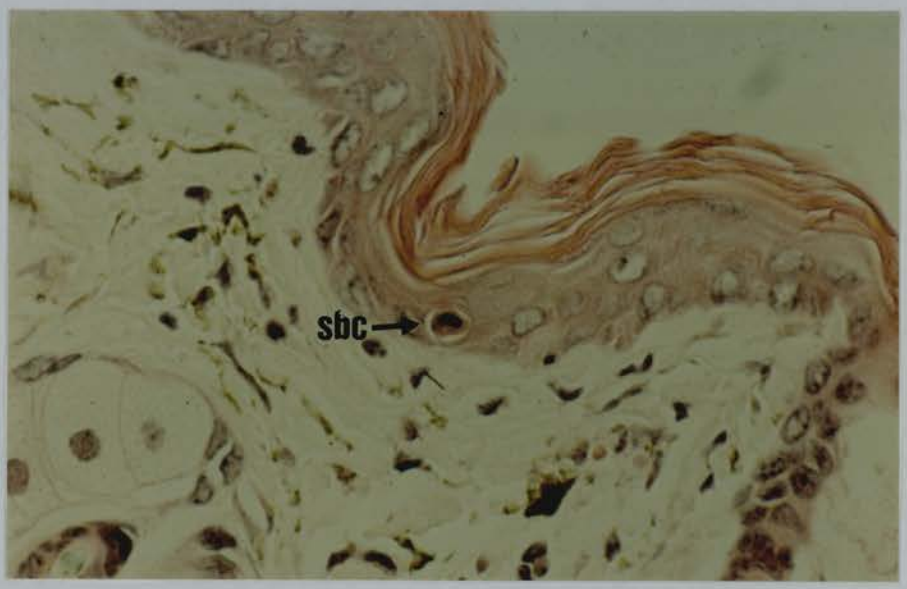

Figure 3.0.3 Sunburn cell in murine epidermis following two weeks of broadband UVB (1000 J/m^2). The glassy, eosinophilic cytoplasm and the pyknotic nucleus can be seen. Magnification x40.

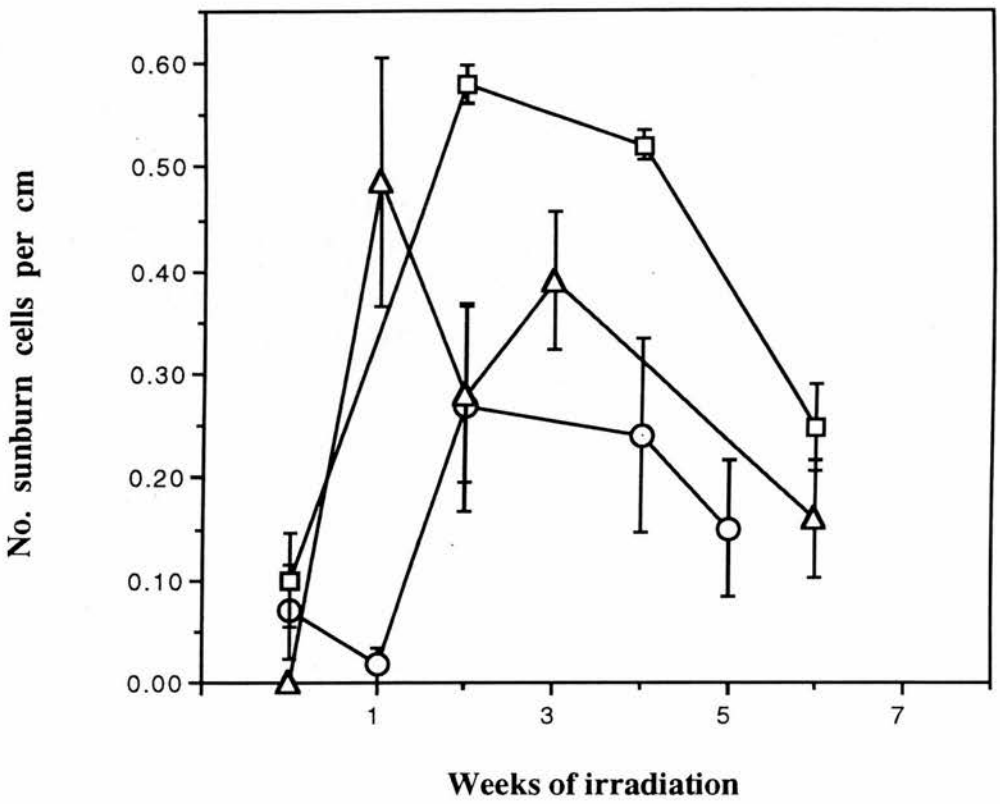

Figure 3.0.4 The number of SBC per cm of interfollicular epidermis following irradiation with 500 J/m^2 UVB (triangles), 1000 J/m^2 UVB (squares) or 3000 J/m^2 TL01 (circles). Bars represent the SEM, n=12. Three different values at time point 0 are presented because three separate control groups were used, one for each irradiation procedure; these values are not significantly different from each other.

obtained following broadband UVB exposure throughout the irradiation period (Figure 3.0.4). After 3 weeks of irradiation, the numbers of SBC started to decline despite continued exposure with all three lamps. The maximum density of SBC obtained after chronic irradiation was 0.58 SBC/cm. In contrast, 24 hr after one acute dose of 1500 J/m^2 UVB, 5.4 SBC/cm were found.

The thickness of the epidermis is an indicator of the response of the skin to UV radiation. Epidermal thickness increased initially after both doses of broadband UVB irradiation (Figure 3.0.5). The overall increase with 1000 J/m^2 UVB was significantly higher than with 500 J/m^2 exposures and led to a doubling of the thickness of the epidermis in these mice. After a slight, but significant (p=0.05) increase, TL01 exposure did not affect epidermal thickness.

3.0.4 The effect of the two UV sources on UCA isomers

Chronic UVB exposure with 500 J/m^2 or 1000 J/m^2 increased the *cis*-UCA concentration in mouse ears from 10 μg/g wet weight of tissue to 54-57 μg/g or 160 μg/g, respectively (Figure 3.0.6). This amount of *cis*-UCA was maintained throughout the six weeks of irradiation and was equivalent to 25% and 38% of total UCA, respectively. Exposure to TL01 was equally efficient at isomerising UCA and 170 μg *cis*-UCA per gram of ear was detected after 1-3 weeks of irradiation. This was equivalent to 35% of total UCA in this experiment. However, after 5 weeks of TL01 exposure the *cis*-UCA concentration was reduced to 100 μg/g (Figure 3.0.6). The total UCA concentration remained relatively constant throughout the 6 weeks of irradiation.

3.0.5 The effect of the two UV sources on the CH response

Six weeks of chronic UVB exposure of the shaved backs of mice with either 500 J/m^2 or 1000 J/m^2 of broadband UVB suppressed the CH response to oxazolone by over 60% (Figure 3.0.7). This was statistically significant in both cases. In contrast, 6 weeks of chronic TL01 exposure (3000 J/m^2) had no effect on the CH response to oxazolone (Figure 3.0.7).

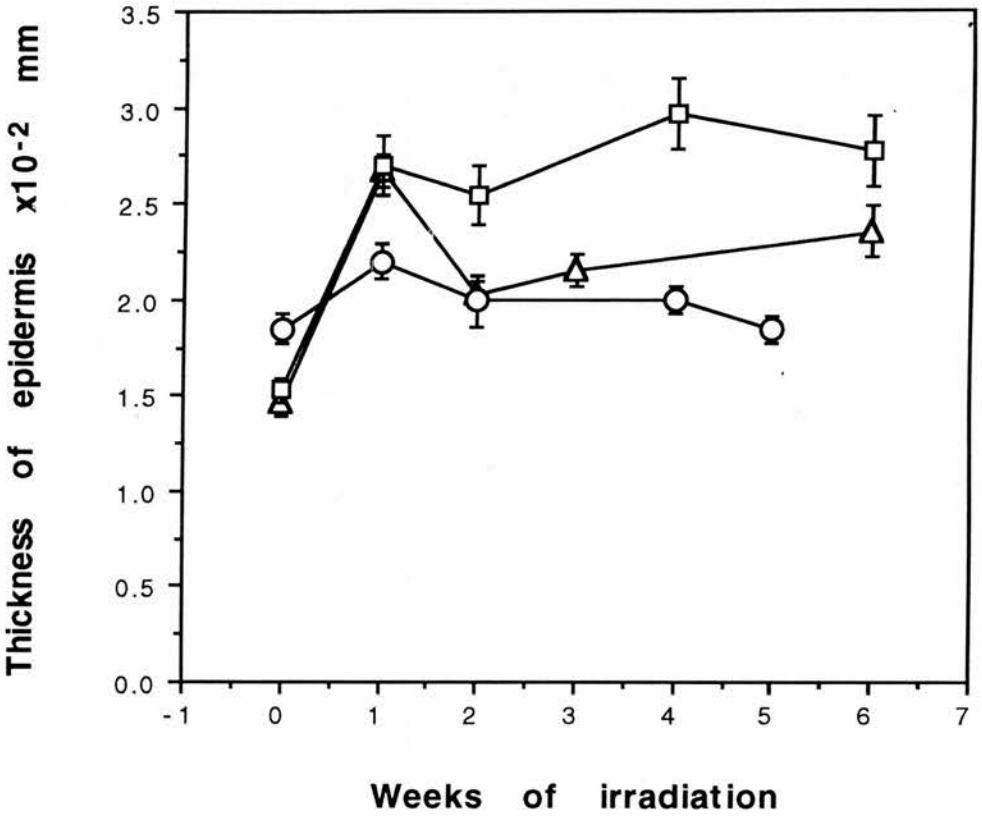

Figure 3.0.5 The thickness of the epidermis following irradiation with 500 J/m^2 UVB (triangles), 1000 J/m^2 (squares) or 3000 J/m^2 TL01 (circles). The bars represent SEM, n is approximately 100. Using the UVB source all values were significantly higher ($p<0.05$) than nonirradiated controls (time point 0), but only the time point at 1 week was significantly higher ($p=0.05$) using the TL01 source.

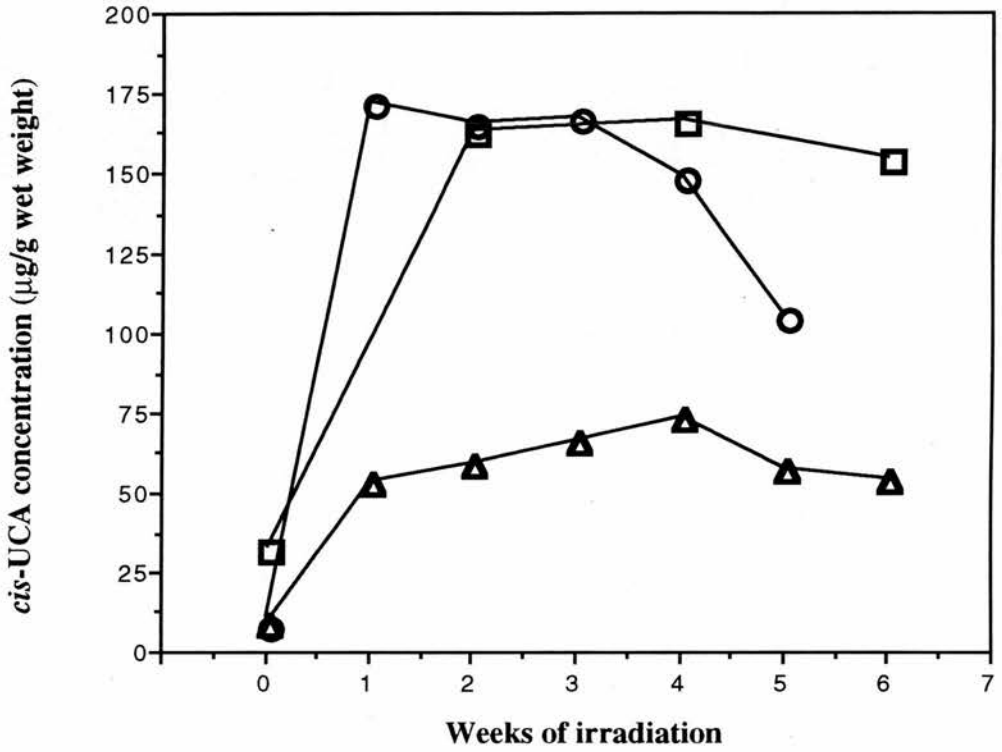

Figure 3.0.6 The *cis*-UCA concentration in C3H/HeN mouse ears following irradiation with 500 J/m^2 UVB (triangles), 1000 J/m^2 UVB (squares) or 3000 J/m^2 TL01 (circles). The standard errors of all the timepoints on the figure were below 22 µg/g, n=3.

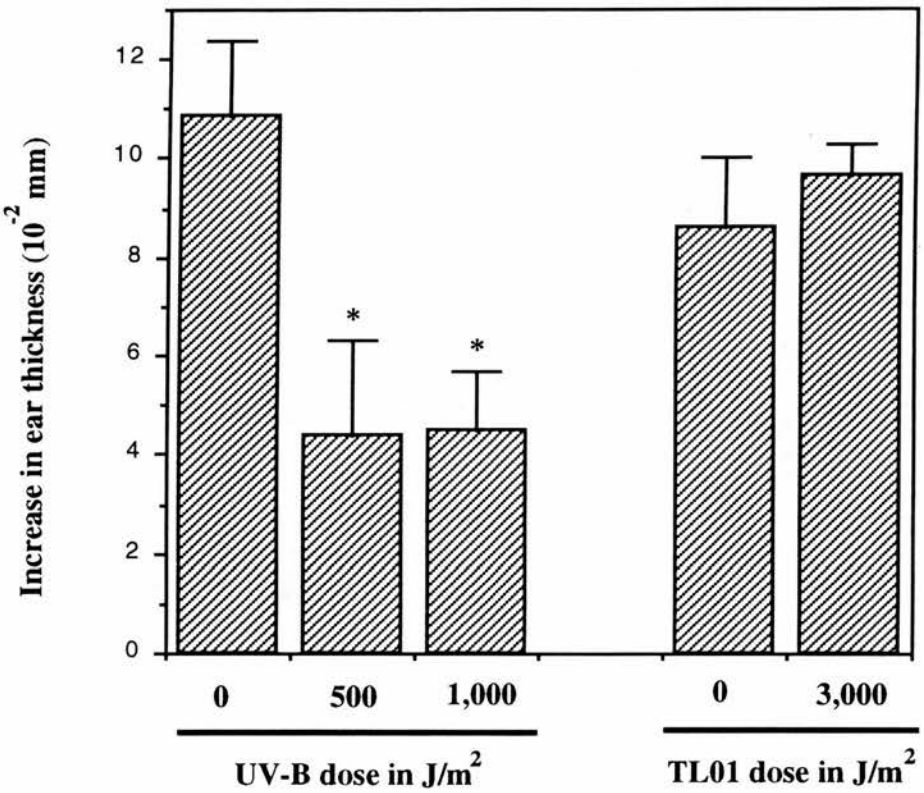

Figure 3.0.7 The CH ear swelling response to oxazolone after 6 weeks of irradiation with UVB and TL01 lamps on shaved dorsal surfaces of mice. The bars indicate the standard error of the mean. *P<0.05 compared with nonirradiated controls, n=9.

3.0.6 Summary of chronic UVB results

A chronic UVB exposure protocol, using broadband (270-350 nm) and narrowband UV (311-312 nm) sources, was used to examine the effect of UVB on the SIS. Exposure to both sources reduced the number of LC in the epidermis. Exposure to the broadband but not the narrowband source induced skin damage, measured by skin thickening, in a dose dependent manner. Although this damage resulted from broadband UVB only, both sources led to a small increase in SBC, which are thought to represent an apoptotic keratinocyte population. Both sources were equally efficient at inducing the conversion of *trans*-UCA to the *cis*-isomer, but only the broadband source suppressed CH responses. Thus LC loss and isomerisation of UCA may not be the primary mediators of immunosuppression during chronic UVB exposure.

In addition, there appeared to be an adaptive response to chronic UVB exposure. LC were not completely eliminated from the epidermis during the six week period. They fell to a minimum at 2-3 weeks of chronic broadband exposure, but numbers were beginning to increase again by week 5. SBC numbers peaked by week two of exposure to both sources, but were barely detectable by week 6. Similarly *cis*-UCA levels peaked at weeks 1-2, but failed to reach the photostationary state (where 50% of the total UCA is in the *cis* form, a point beyond which no further isomerisation takes place), during the 6 weeks of exposure. This adaptation to chronic UV exposure is possibly related to the tanning and increased epidermal thickness induced by the broadband source. However, though the narrowband source failed to induce either tanning or thickening, the skin of mice exposed to this source showed similar adaptive responses to those mice exposed to broadband UVB. Hence, narrowband UVB failed to completely deplete LC, failed to induce the isomerisation of UCA to the photostationary point and showed a reduced ability to induce SBC by 4-6 weeks of exposure. Therefore it is possible that other adaptive mechanisms exist, which are separate from the development of pigmentation or skin thickening.

3.1. The effect of UVB on DC function and phenotype

3.1.1 Introduction

As described in the introduction cutaneous exposure to contact sensitisers induces the migration of antigen-bearing LC from the epidermis into the afferent lymph and their accumulation as immunostimulatory DC in the DLN (Kripke *et al.*1990; Macatonia *et al.*1986). During migration, LC are subject to changes in both phenotype and function. They become efficient antigen presenting cells and acquire the ability to cluster with, and activate, naive T lymphocytes (Inaba *et al.*1984). The ability of DC to induce the expansion of an effector T cell population from naive T cells in the DLN is required for the initiation of primary immune responses. The changes that DC undergo during their migration to the DLN are thought to be mirrored by those induced by culture of freshly isolated epidermal LC *in vitro* for several days in the presence of keratinocytes or appropriate epidermal cytokines (Romani *et al.*1989).

Irradiation with ultraviolet B light (UVB) compromises primary cutaneous immune responses in certain strains of mice. These changes correspond to the induction of anergy or antigen-specific unresponsiveness to a variety of antigens, including skin allergens (Streilein and Bergstresser, 1988). It has been known for many years that following UVB irradiation, there is a reduction in the number of LC in the epidermis (Toews *et al.*1980). In addition, an increased number of DC accumulate in lymph nodes draining the site of exposure (Moodycliffe *et al.*1992). *In vitro* studies have shown clearly that UVB is able to inhibit culture-induced expression of a variety of important LC surface molecules, and their activity as antigen-presenting cells (Tang and Udey, 1991). Primary cutaneous immune responses are induced in lymph nodes draining the skin, and lymph node DC are considered to be essential for the stimulation of naive antigen-responsive T lymphocytes. Therefore, it was decided to examine whether the irradiation of C3H/HeN mice with doses of UVB which were sufficient to cause the suppression of both CH (Moodycliffe *et al.*1994) and DH responses (Ross *et al.*1986), were associated with changes in the function or phenotype of DC in the

DLN. The function of the DC was analysed in two ways. First, the ability of oxazolone and FITC-sensitised DC from control and irradiated mice to initiate antigen specific secondary proliferative responses by LNC from sensitised mice was measured. Second, the ability of DC from unirradiated and irradiated mice to act as allostimulators in primary mixed lymphocyte reactions (MLR) was examined. In addition, the phenotypic characteristics of the DC in DLN from irradiated and unirradiated skin were compared. Three determinants were analysed, namely MHC class II, ICAM-1 and B7-2. MHC class II was examined due to its role in presentation of antigen to $CD4^+$ T lymphocytes. ICAM-1 is an adhesion molecule known to facilitate the antigen-independent interaction between DC and T lymphocytes (Van Seventer *et al.* 1992). Furthermore, expression by LC of this determinant may be important in guiding their directed movement to, and accumulation in, DLN (Ma *et al.* 1994). B7-2 (CD86) provides an important second signal for T cell activation (Lanier *et al.* 1995).

3.1.2 Immunosuppression following acute UVB-exposure

C3H/HeN mice have been categorised as being susceptible to the effects of UVB in local and systemic models of immunosuppression (Streilein and Bergstresser, 1988; Noonan and Hoffman, 1994). In addition, exposure to the doses of UVB used widely in this study (1440 J/m^2 UVB 48 and 24 hrs prior to sensitisation) caused a 50% suppression of CH responses in C3H/HeN mice in previous studies (Moodycliffe *et al.* 1994). In order to confirm that C3H/HeN mice were susceptible to the effects of low-dose UVB, CH responses were carried out. Figure 3.1.1 shows the results from the first experiment. Exposure to UVB caused a non significant (p=0.05), 31% suppression of the CH response. In Figure 3.1.2 the same CH protocol was carried out except that the ears of mice were covered during irradiation. Using the new protocol, UVB suppressed CH responses by 46%, a significant reduction (p=0.01)

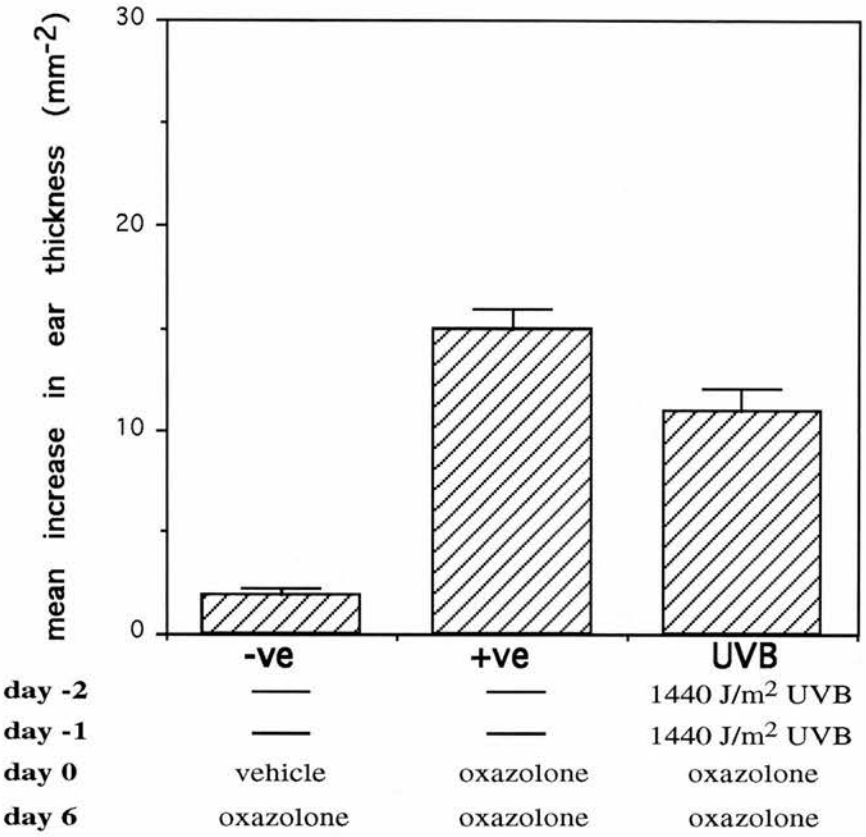

Figure 3.1.1 Suppression of CH response to oxazolone by UVB in C3H/HeN. Mice were exposed to UVB (2x1440 J/m^2) or were unirradiated (-ve and +ve) prior to sensitisation with oxazolone (+ve and UVB) or vehicle on the irradiated site. The ears of the mice were uncovered during exposure to UVB. Six days later the ears of all mice were challenged with oxazolone and the results expressed as the mean increase in ear swelling in mm^{-2}± SEM (n=7).

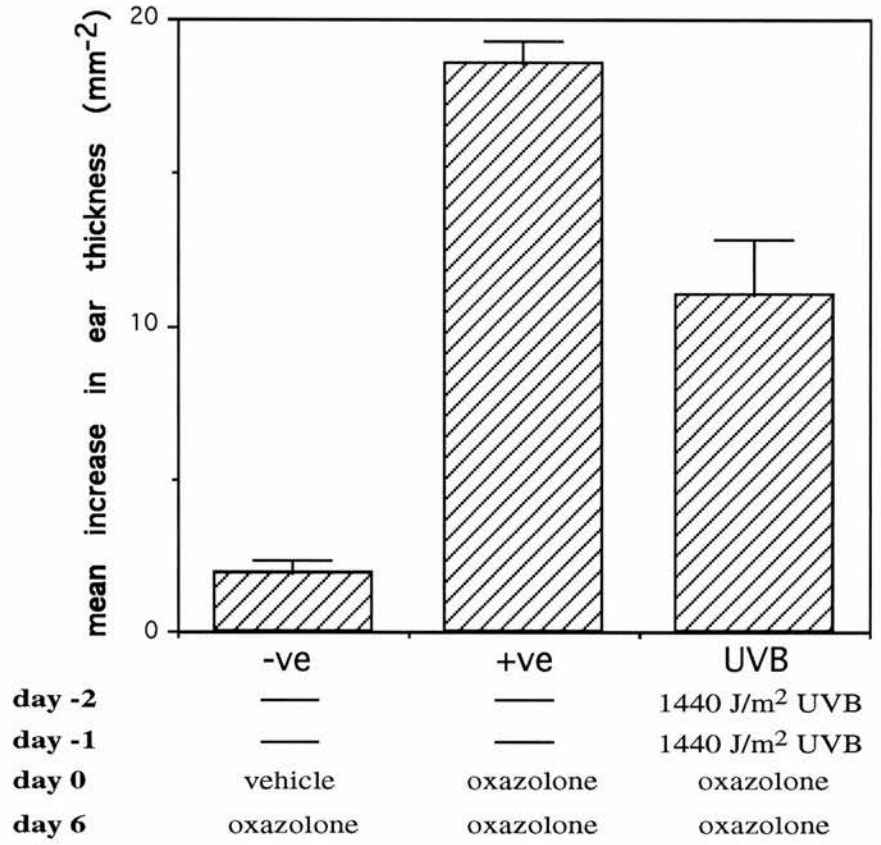

Figure 3.1.2. Suppression of CH response to oxazolone by UVB in C3H/HeN. Mice were exposed to UVB (2x1440 J/m^2) or were unirradiated (-ve and +ve) prior to sensitisation with oxazolone (+ and UVB) or vehicle on the irradiated site. During the irradiation the ears of mice were protected from UVB. Six days later the ears of all mice were challenged with oxazolone and the results expressed as the mean increase in ear swelling in mm^{-2}± SEM (n=7).

compared with the positive control.

3.1.3 Identification of hapten-bearing DC in the DLN of sensitised mice

To determine whether the accumulation of DC in the DLN after sensitisation could be attributed to an influx of DC from the skin, mice were painted on the dorsum of both ears with FITC or oxazolone. Eighteen hrs later draining auricular lymph nodes were removed and DC^- and DC^+ populations prepared. Background fluorescence levels were set at 1% on DC^- and DC^+ populations from oxazolone sensitised mice. Around 28% of LNC in the DC^- population displayed FITC on their surface, but the intensity of staining was low with a mean fluorescence of 1.12 on a $\log_{10}$ scale (histogram not shown). Around 37% of all the cells in the DC enriched population had FITC on their surface and the intensity of staining was high with a mean fluorescence of 24.7 on a $\log_{10}$ scale (Figure 3.1.3a). Of the cells with DC characteristics (the large granular population), 73% bore FITC (Figure 3.1.3b).

3.1.4 The effect of UVB on the antigen specific accessory activity of DC

3.1.4.1 The ability of FITC-bearing DC to stimulate proliferative responses

The aim of the initial experiment was to determine whether FITC-bearing DC could stimulate antigen-specific proliferation of FITC-sensitised LNC. Figure 3.1.4 shows the results from this experiment. It can be seen that DC from untreated mice were poor stimulators of proliferative responses, whether they were used to stimulate unsensitised or FITC-sensitised LNC. In contrast, DC from FITC-sensitised mice were significantly ($p=0.01$) better than naive DC at stimulating FITC-sensitised and unsensitised LNC. Although there was no difference in the background proliferation between sensitised and non-sensitised LNC populations, FITC-sensitised DC showed

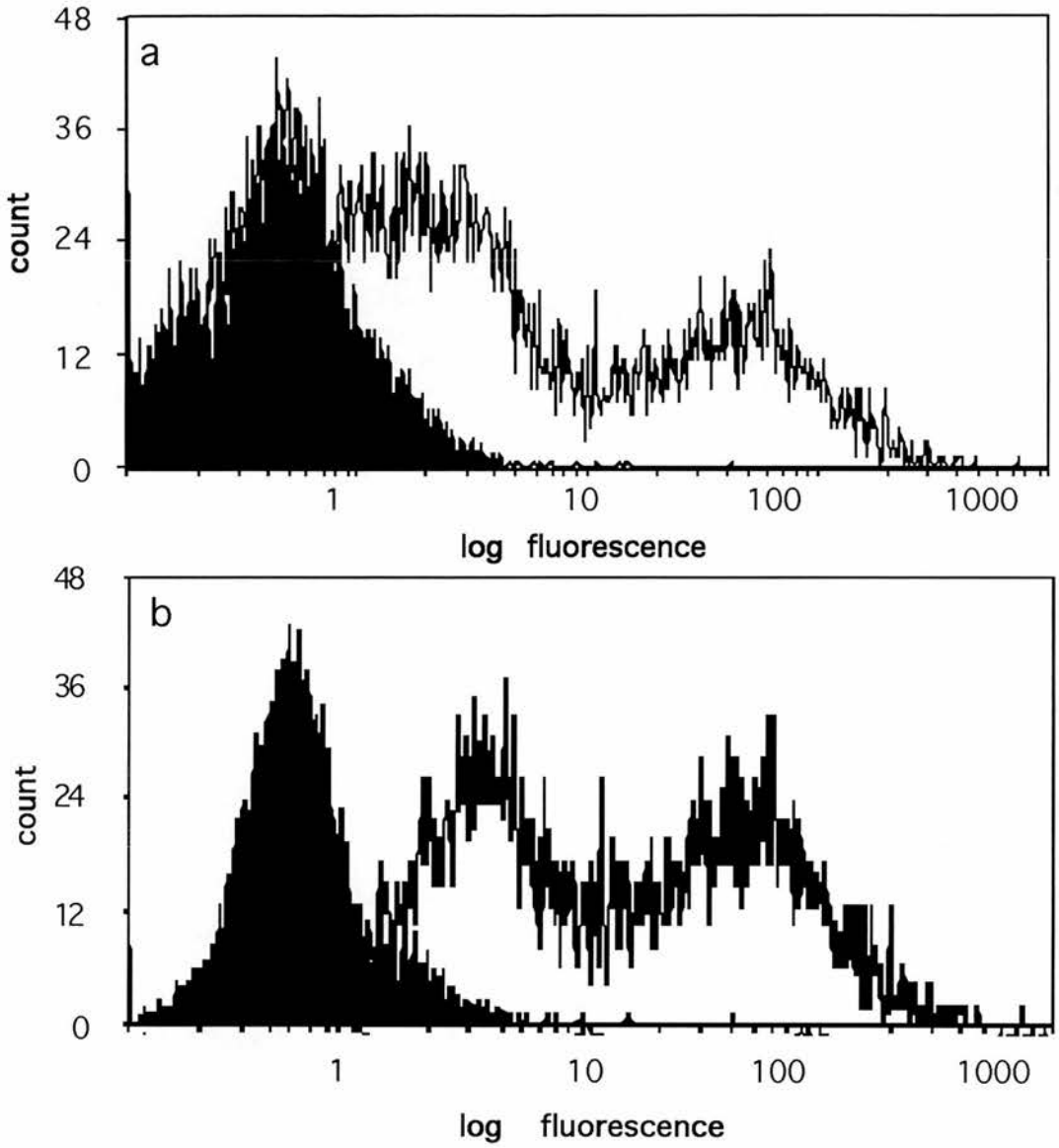

Figure 3.1.3 The expression of FITC on DC enriched from the DLN following skin painting with FITC. Mice (n=10) were ear painted with oxazolone or FITC. 18 hrs later enriched oxazolone (filled area) and FITC (open area) sensitised DC populations were prepared from lymph nodes and their fluorescence was analysed using a flow cytometer. Figure 3.1.3.a shows the fluorescence on all cells within the enriched cell population. Figure 3.1.3.b shows fluorescence on the cells within the DC region.

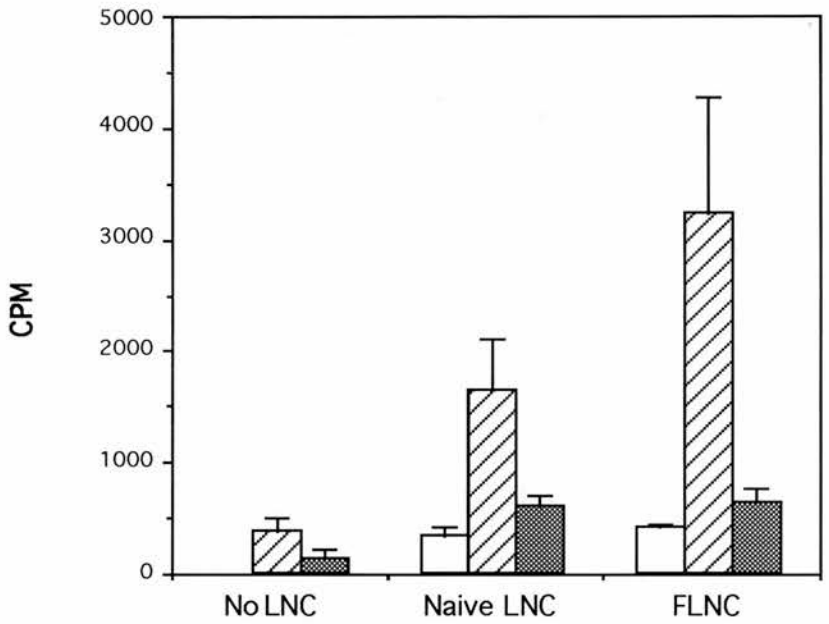

Figure 3.1.4 The ability of DC from lymph nodes draining FITC-sensitised or unsensitised sites to stimulate lymphoproliferation *in vitro*. Responder LNC from unsensitised mice (Naive LNC) or FITC-sensitised mice (FLNC) were cultured alone (□), with FITC-bearing DC (▨) or naive DC (▩). LNC were prepared from groups of 3 mice, and DC from groups of 10 mice. DC were added at a stimulator:responder ratio of 1:55. The cells were cultured for 48 hours and ^{3}H-methyl thymidine was added to all wells 18 hours prior to termination of culture. The results are expressed as mean CPM±SD for 5 replicate cultures.

spontaneous proliferation of around 400 CPM compared with approximately 150 CPM for naive DC.

3.1.4.2 The ability of UVB to suppress DC-induced proliferative responses

Having shown that FITC-bearing DC could induce lymphoproliferation, it was decided to examine the effect of UVB on the system. In this experiment the concentration of FITC used to sensitise animals was reduced from 5% to 1% and in addition the antigen specificity of DC and the UVB-induced suppression of CH responses to oxazolone was examined by sensitising some groups of responder and stimulator mice with 0.25% oxazolone. It was decided to reduce the concentration of FITC used because when used at 5% the FITC tended to cake onto the ears and provoked intense washing in the mice. The net effect of this was to spread the FITC around both the mouse and the cage. Using a lower concentration of FITC minimised this problem. The effect of UVB on this system was examined by exposing certain groups of stimulator mice to a single dose of UVB (1440 J/m^2) 24 hrs prior to the sensitisation. DC from these mice and from mice that were not irradiated prior to sensitisation, were used as accessory cells.

It can be seen that a single dose of UVB failed to suppress the ability of hapten-bearing DC to stimulate proliferation (Figure 3.1.5). FITC-bearing DC from irradiated mice induced twice as much proliferation of FITC-sensitised LNC and oxazolone sensitised LNC as FITC-bearing DC from unirradiated control animals. oxazolone-sensitised DC from irradiated mice induced slightly more proliferation from oxazolone and FITC-sensitised LNC as oxazolone-sensitised DC from unirradiated control mice. However, oxazolone-sensitised DC performed poorly as APC, lacking potency in the induction of proliferative responses and failing to show antigen specificity.

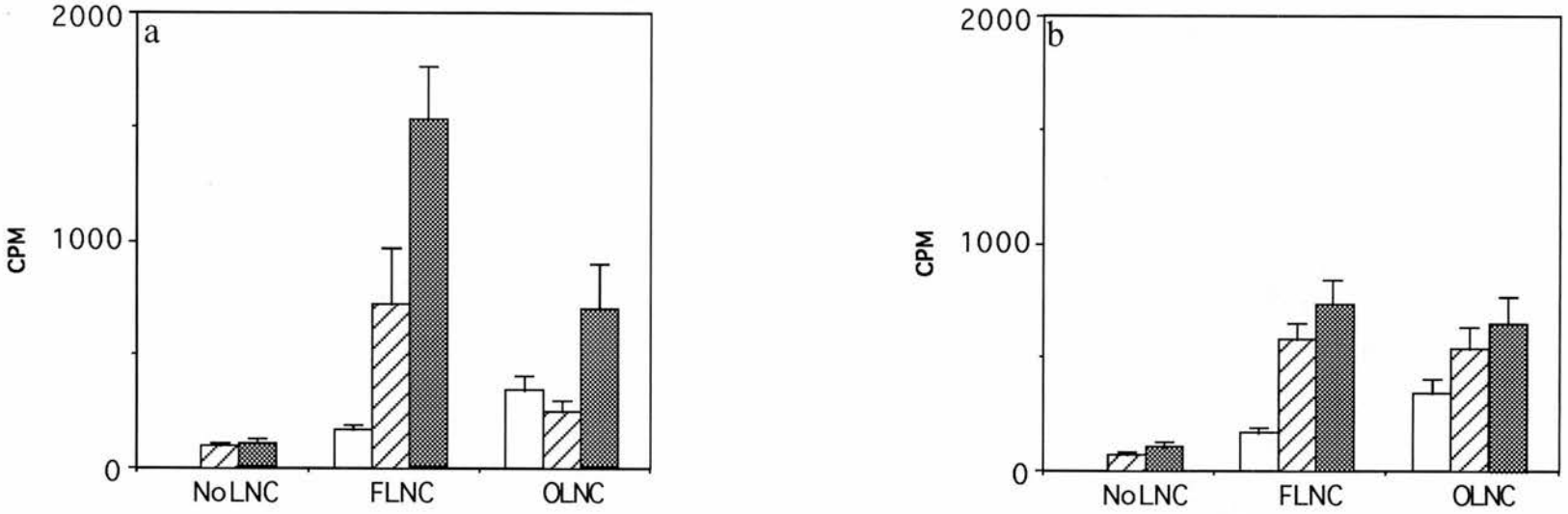

Figure 3.1.5 The effect of UVB on the ability of DC enriched from mice sensitised with FITC or oxazolone to stimulate FITC and oxazolone specific lymphoproliferation *in vitro*. In Figure 3.1.5a, FITC- (FLNC) and oxazolone- (OLNC) sensitised responder LNC (from 5 mice per group) were cultured alone (□), with FITC-bearing DC from unirradiated mice (n=10) (▨), or with FITC bearing DC from mice (n=10) exposed to UVB (1440 J/m^2 total) prior to skin painting (▩) . Figure 3.1.5b is the same except that oxazolone-sensitised DC from unirradiated mice (n=10) (▨), or oxazolone-sensitised DC from mice (n=10) exposed to UVB prior to skin painting (▩) were used instead of FITC-bearing DC. In both experiments DC were added at a stimulator:responder ratio of 1:55. The cells were cultured for 48 hours and ^{3}H-methyl thymidine was added to all wells 18 hours prior to termination of culture. The results are expressed as mean CPM±SD for 5 replicate cultures.

3.1.4.3 Changes in the protocol to improve the specificity and magnitude of proliferative responses

Because of the lack of antigen specificity and the low proliferative responses shown in Figures 3.1.4 and 3.1.5, a number of changes were made to the protocol. In initial experiments responder and stimulator lymph nodes were harvested at the same time and LNC suspensions were prepared. This meant that suspensions that were to be enriched for DC sat on ice while responder populations were counted and plated out. To reduce the time between removal of lymph nodes and the addition of enriched DC to cultures, the protocol was changed. In all the following experiments, responder lymph nodes and stimulator lymph nodes were prepared separately. Responder lymph nodes were harvested first, processed into LNC suspensions and seeded into wells. Once the first stage was completed, stimulator lymph nodes were harvested and enriched DC cell suspensions were prepared. In this way DC processing time was minimised. Further changes to the protocol included raising the concentration of responder cells from $1.25x10^5$ to $2.5x10^5$ cells/well, DC were still added at a 1:55 stimulator: responder ratio unless otherwise stated. Finally the concentration of FITC was raised to 2.5%, in order to increase the magnitude of proliferative responses, as it was noted that proliferation fell when 1% FITC (Figure 3.1.5) was used instead of 5% FITC (Figure 3.1.4) to sensitise mice. FITC was used at 2.5% in all subsequent experiments.

Figure 3.1.6 shows the results produced using the changed protocol. The reduced processing time may be reflected in the increased hapten specificity of responses. In this experiment both FITC and oxazolone-sensitised DC are significantly ($P \leq 0.01$) better at inducing hapten-specific proliferative responses than they are at inducing non-specific proliferative responses. FITC-bearing DC fail to induce a significant increase in the proliferation of oxazolone-sensitised LNC over background counts. However oxazolone-sensitised DC showed some lack of

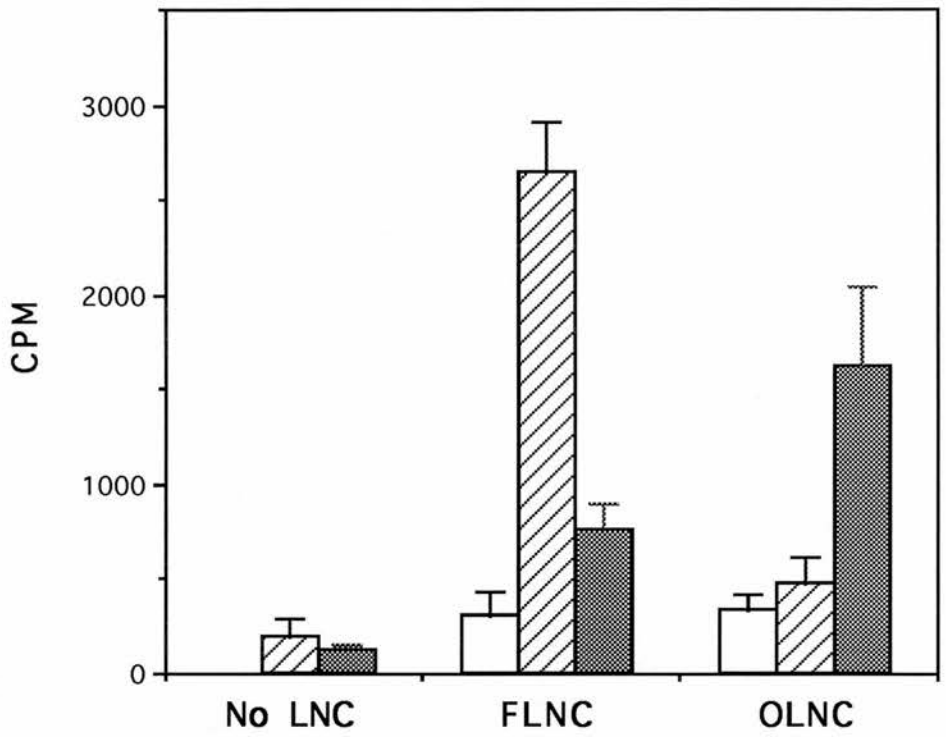

Figure 3.1.6 The ability of DC from lymph nodes draining FITC- or oxazolone- sensitised sites to stimulate specific lymphoproliferation *in vitro.* Responder LNC from FITC-sensitised mice (n=3) (FLNC) or oxazolone-sensitised mice (n=3) (OLNC) were cultured alone (□), or with FITC-(▨) or oxazolone-(▩) sensitised DC (10 mice per group). DC were added at a stimulator:responder ratio of 1:55. The cells were cultured for 48 hours and ^{3}H-methyl thymidine was added to all wells 18 hours prior to termination of culture. The results are expressed as mean CPM±SD for 5 replicate cultures.

specificity, causing a significant (p=0.01) increase in the proliferative responses of FITC-sensitised LNC, compared with background counts.

3.1.4.4 The effect of UVB on DC function using the new protocol

In the previous experiment the revised protocol provided a population of DC that was capable of inducing antigen-specific proliferative responses. Therefore, using the same protocol, the effect of UVB on the ability of DC to induce proliferation was examined. Previously stimulator mice had been exposed to a single dose of UVB (1440 J/m^2) 24 hrs prior to sensitisation. In this experiment two consecutive doses were used, 1440 J/m^2 at -48 and -24 hrs prior to sensitisation. This exposure has been shown to suppress CH responses by around 50% in C3H/HeN mice (Moodycliffe *et al.* 1994), and has suppressed CH responses in C3H/HeN mice to a similar extent in my hands (Figure 3.1.2). Figures 3.1.7a and 3.1.7b show the results from this experiment. In Figure 3.1.7 a, FITC-bearing DC from normal and irradiated mice were used to stimulate the proliferation of FITC and oxazolone-sensitised LNC. Exposure of mice to UVB did not reduce significantly the ability of DC to induce FITC-specific proliferation. In addition, DC from irradiated animals were significantly (p=0.025) better at inducing non-specific proliferation from oxazolone-sensitised LNC.

In Figure 3.1.7b, oxazolone-sensitised DC were used instead of FITC DC. A notable feature of these data is the lack of immunostimulatory activity of the oxazolone-sensitised DC population. These DC failed to induce proliferative responses above background levels. The failure of the oxazolone DC may have been due to a problem in the enrichment stage, that yielded between 9-18x10^3 DC per LN for the other DC populations, but only 2700 DC per LN from oxazolone-sensitised mice. Although this provided enough DC to use in the assay, the unusually low numbers raise doubts about the quality of the enrichment. Due to the inability of the oxazolone-sensitised DC population to induce proliferation, the UVB-treated DC population

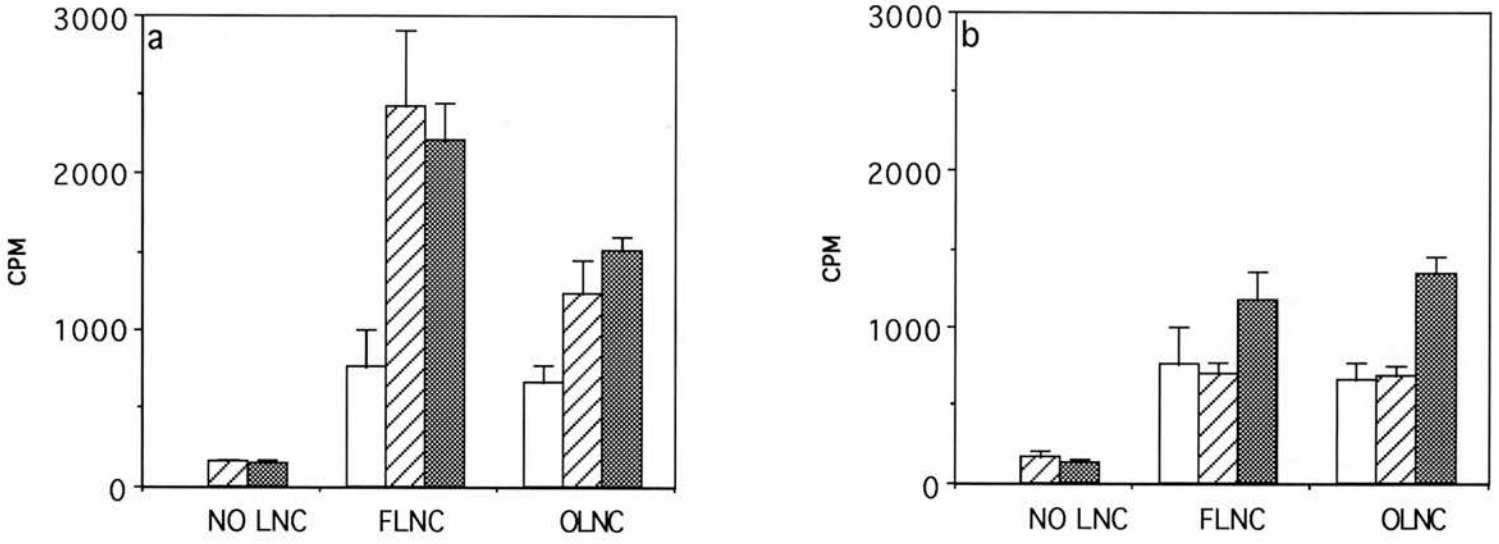

Figure 3.1.7 The effect of UVB on the ability of DC enriched from lymph nodes of mice sensitised with oxazolone or FITC to stimulate hapten specific lymphoproliferation *in vitro.* In Figure 3.1.7a, FITC (FLNC) and oxazolone (OLNC) sensitised responder LNC (3 mice per group) were cultured alone (□), with FITC-bearing DC from unirradiated mice (n=10) (▨), or with FITC-bearing DC from mice (n=10) exposed to UVB (1440 J/m^2 , -48 and -24 hrs) prior to skin painting (▩). Figure 3.1.7b is the same except that oxazolone-sensitised DC from unirradiated mice (n=10) (▨), or oxazolone-sensitised DC from mice (n=10) exposed to UVB prior to skin painting (▩) were used instead of FITC bearing DC. In both experiments DC were added at a stimulator:responder ratio of 1:55. The cells were cultured for 48 hours and ^{3}H-methyl thymidine was added to all wells 18 hours prior to termination of culture. The results are expressed as mean CPM±SD for 5 replicate cultures.

induced significant increases in antigen-specific (p=0.01) and non-specific (p=0.01) proliferative responses.

The other possibility is that the concentration of oxazolone selected was too low. In this experiment (Figure 3.1.7) and the previous one (Figure 3.1.6) oxazolone was used to sensitise stimulator and responder mice at a concentration of 0.25%. Since both experiments feature poor oxazolone responses, it is possible that this concentration of oxazolone is failing to sensitise responder mice. A feature of this experiment (Figure 3.1.7) but not the previous experiment (Figure 3.1.6) was the lack of DC accumulation in the DLN of oxazolone-sensitised mice. This may have had an effect on the accessory activity of these DC.

3.1.4.5 Titration of DC function

In the previous two experiments the culture of FITC-specific immune responses were larger and more-antigen specific than oxazolone-specific response. It was decided therefore to limit the three following experiments to measurements of responses to FITC.

Initially, the proliferative responses were optimised by altering the numbers of DC and responder cells in the assay. Figure 3.1.8 shows the ability of FITC-bearing DC^+ to stimulate the proliferation of FITC sensitised LNC. Increasing the ratio of stimulator DC to responder cells results in an increase in proliferative responses dependent on DC numbers. In addition, doubling the number of responder cells and DC^+ per well caused increased proliferative responses. When DC-depleted populations were used as stimulator cells, they failed to induce proliferative responses over background. From this experiment it was decided to double the number of responder LNC to $5x10^5$ cells/well, and to add DC at a 1:55 stimulator:responder ratio. This protocol was adopted in all subsequent experiments described in this section.

It is apparent from the data in Figure 3.1.8 that proliferative responses were generally higher compared with those in the preceding experiment. The sensitised

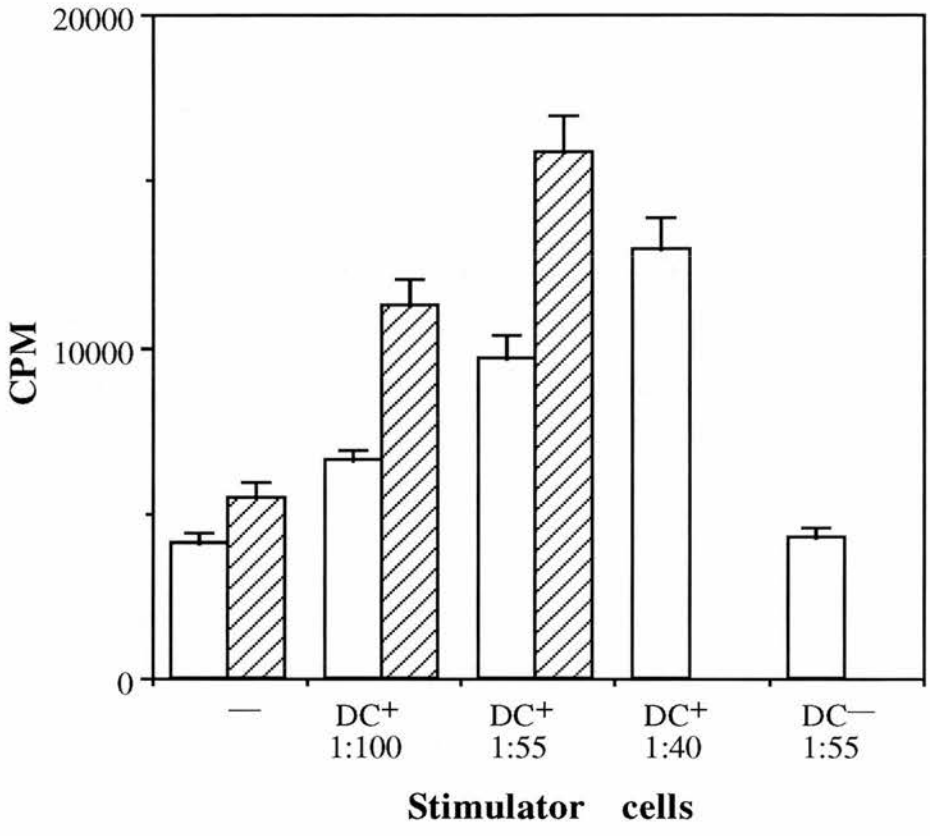

Figure 3.1.8 The ability of DC enriched from mice sensitised with FITC to stimulate FITC-specific lymphoproliferation *in vitro*. FITC-sensitised responder LNC (from 5 mice) were cultured at two concentrations $2.5x10^5$ (□) and $5x10^5$ (▨) cells/well. The LNC were cultured alone (—), or with FITC- bearing DC (DC^+) (from 15 mice) at a variety of stimulator: responder ratios (1:100, 1:55 and 1:40). Responder LNC at $2.5x10^5$ were cultured with DC depleted populations ($DC^—$) at a stimulator:responder ratio of 1:55. The cells were cultured for 48 hours and ^{3}H-methyl thymidine was added to all wells 18 hours prior to termination of culture. The results are expressed as mean CPM±SD for 5 replicate cultures. Responses for DC cultured alone were below 500 CPM.

LNC were harvested on day 6 rather than day 7 following FITC sensitisation and show higher background or spontaneous proliferative responses than in the previous experiments. This may be attributable to the presence of higher numbers of activated cells within the lymph node because of the early harvest.

3.1.4.6 The effect of UVB on FITC-specific responses

Figure 3.1.9 shows the results of two independent experiments in which enriched FITC-DC isolated from control and UVB irradiated mice were used to stimulate the proliferation of LNC from FITC-sensitised mice. In the first experiment (Figure 3.1.9a) there was no significant difference between the accessory cell activity of DC isolated from control or irradiated mice. Similarly in the second experiment (Figure 3.1.9b), the accessory activity of DC was not significantly affected following exposure to UVB *in vivo*.

3.1.4.7 The effect of limiting DC numbers on oxazolone specific responses

Since UVB failed to modulate responses to FITC, its effect on responses to oxazolone were studied. In this experiment DC were used at 2 stimulator:responder concentrations, namely 1:55 and 1:100. It was decided to use DC at a concentration of 1:100 because it was thought possible that using DC at 1:55 was providing super-optimal stimulation of LNC, and masking therefore, conceivable differences between the DC populations. In previous experiments the proliferative responses of oxazolone-sensitised LNC were smaller than FITC-sensitised LNC. Therefore the concentration of oxazolone used to sensitise mice was raised from 0.25% to 1%. In addition, the concentration of responder LNC was increased to $5x10^5$ cells/well. In Figure 3.1.10, DC were used at 2 stimulator:responder ratios, 1:100 and 1:55, and the antigen specificity of the response was examined by using oxazolone- and FITC-sensitised DC as stimulator cells for LNC from oxazolone-sensitised mice.

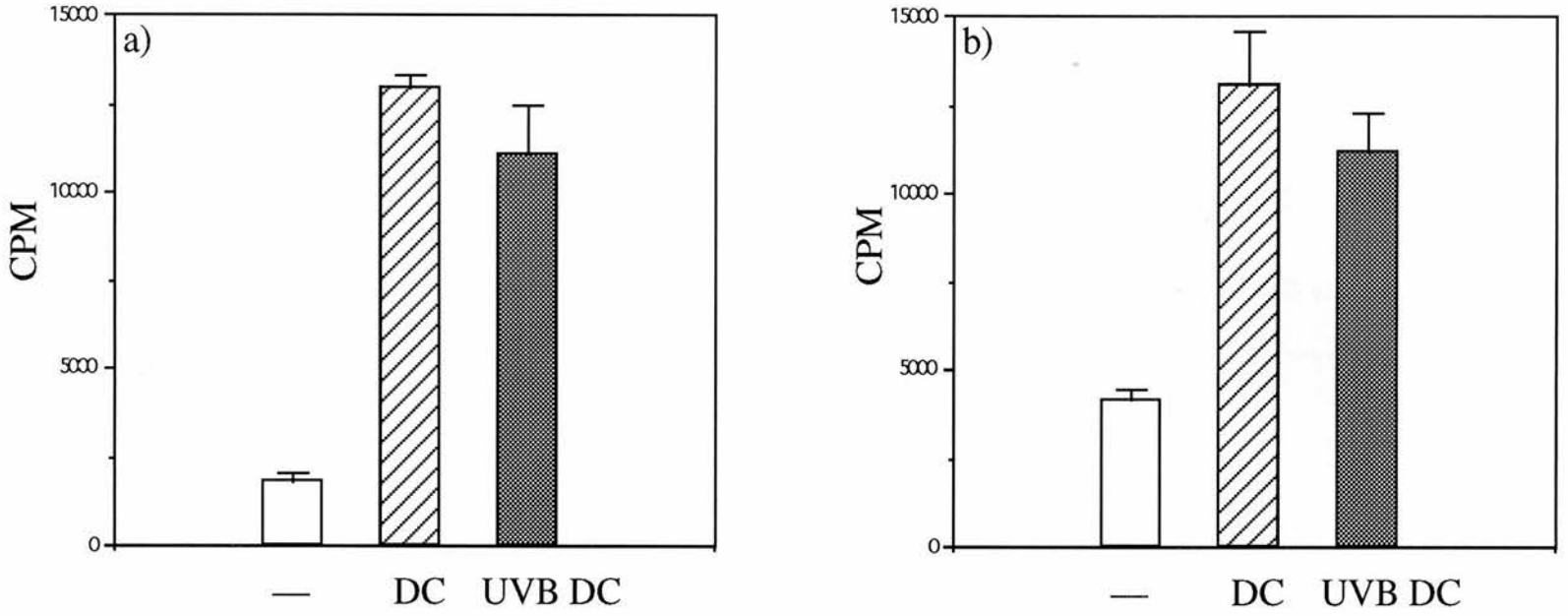

Figure 3.1.9 The effect of UVB on the ability of DC enriched from mice sensitised with FITC to stimulate FITC-specific lymphoproliferation *in vitro*. The results from two separate experiments are shown in Figure 3.1.9a and 3.1.9b. FITC sensitised responder LNC (from 3 mice) were cultured alone (□), with FITC-bearing DC from unirradiated mice (n=10) (▨), or FITC-bearing DC from mice (n=10) exposed to UVB (1440 J/m^2, -48 and -24 hrs) prior to skin painting (▩). DC were cultured at a stimulator:responder ratio of 1:55. The cells were cultured for 48 hours and ^{3}H-methyl thymidine was added to all wells 18 hours prior to termination of culture. The results are expressed as mean CPM±SD for 5 replicate cultures. The responses for DC cultured alone were below 500 CPM.

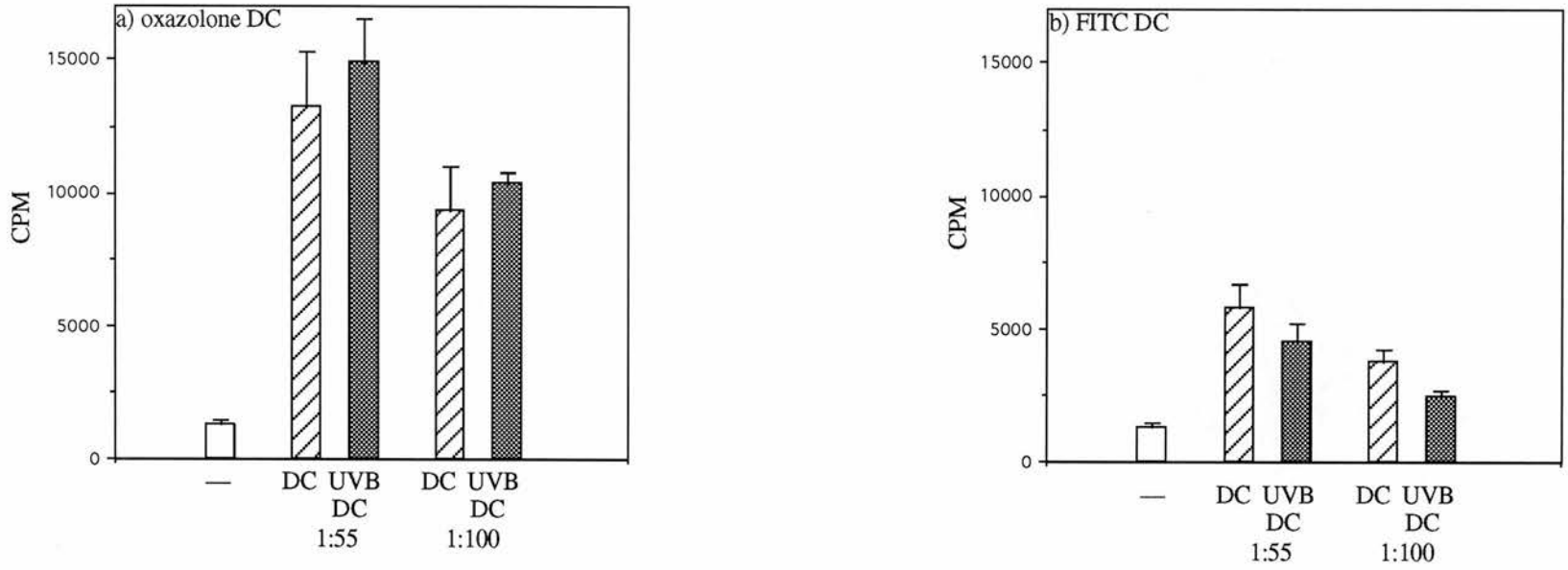

Figure 3.1.10 The effect of UVB on the ability of DC enriched from mice sensitised with oxazolone or FITC to stimulate oxazolone-specific lymphoproliferation *in vitro*. In Figure 3.1.10.a, oxazolone-sensitised responder LNC (from 3 mice) were cultured alone (□), with oxazolone-sensitised DC from unirradiated mice (n=10) (▨), or oxazolone-sensitised DC from mice (n=10) exposed to UVB (1440 J/m^2, -48 and -24 hrs) prior to skin painting (▩). Figure 3.1.10b is the same except that FITC-bearing DC from unirradiated mice (n=10) (▨), or FITC-bearing DC from mice (n=10) exposed to UVB prior to skin painting (▩) were used instead of oxazolone-sensitised DC. The cells were cultured for 48 hours and ^{3}H-methyl thymidine was added to all wells 18 hours prior to termination of culture. The results are expressed as mean CPM±SD for 5 replicate cultures. Responses for DC cultured alone were below 500 CPM.

Figure 3.1.10a shows the proliferative response of LNC cultured in the presence of oxazolone-sensitised DC from control and irradiated mice. Oxazolone-sensitised DC stimulated good proliferative responses by LNC from mice sensitised with oxazolone in a dose-dependent manner. UVB treatment did not inhibit this ability. The failure of UVB to affect the accessory activity of DC was seen when DC were present at both stimulator:responder ratios. The hapten specificity of the response is shown in Figure 3.1.10b. Although FITC-sensitised DC were able to induce limited antigen non-specific proliferative responses from oxazolone-sensitised LNC, their capacity to induce proliferation was poor when compared with the antigen specific proliferative responses illustrated in Figure 3.1.10a.

3.1.4.8 The effect of UVB on DC function: titration of DC concentration

In order to examine whether UVB would influence proliferative responses over a range of stimulator:responder ratios, mice were either left unirradiated or were irradiated (1440 J/m^2, -48 and -24 hrs) prior to FITC- or oxazolone sensitisation. Eighteen hrs after sensitisation DC were enriched from the DLN. These DC were then cultured with FITC-sensitised LNC at 4 stimulator:responder ratios of (1:20, 1:40, 1:55, and 1:100). Oxazolone-sensitised DC were not used at 1:20 due to lack of cell numbers.

In this particular experiment, the responder LNC were harvested on day 8 rather than day 7, which may explain the low proliferative responses which were obtained (Figure 3.1.11). However, as in previous experiments, it can be seen that exposure to UVB failed to affect DC accessory function in this assay. At all concentrations except 1:100, FITC-bearing DC from control and irradiated mice showed no significant difference in their ability to induce proliferation from FITC-sensitised DC. At the stimulator:responder ratio of 1:100, DC from irradiated mice were significantly ($p \leq 0.01$) better at inducing proliferation, though the difference in counts was small. Oxazolone-sensitised DC, which were used at a single concentration

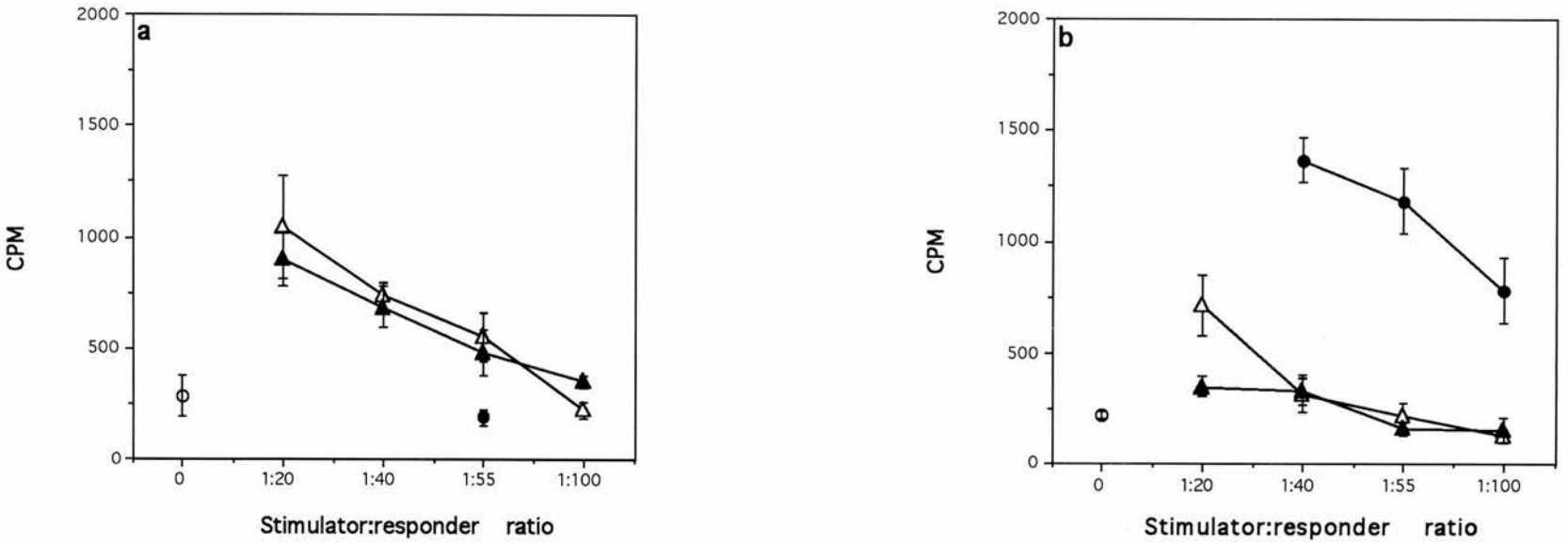

Figure 3.1.11 The effect of UVB on the ability of FITC-bearing DC to induce FITC specific lymphoproliferation *in vitro*. In Figure 3.1.11a FITC-sensitised LNC (from 5 mice) were cultured alone (O), or with hapten bearing DC at a variety of stimulator to responder ratios. FITC-bearing DC were enriched from unirradiated (Δ) and UVB irradiated (1440J/m^2 , -48 and -24 hrs) mice (n=15) (▲), while oxazolone bearing DC were enriched from unirradiated mice (n=15) only (●). In Figure 3.1.11b oxazolone-sensitised LNC (from 5 mice) were cultured alone (O), or with the same DC populations used in figure 3.1.11.a. The cells were cultured for 48 hrs and ^{3}H-methyl thymidine was added to all wells 18 hours prior to the termination of culture. The results are expressed as the mean CPM±SD for 5 replicate cultures. The proliferative responses of DC alone have not been shown but have been subtracted from the appropriate groups.

(1:55) in Figure 3.1.11, stimulated less proliferation from FITC-sensitised LNC than either FITC-bearing DC population.

Figure 3.1.11b shows the specificity of the response with all except the highest concentration (1:20 stimulator:responder ratio) of FITC-bearing DC from the control group only, failing to induce proliferative responses from oxazolone-sensitised LNC. As expected, oxazolone-sensitised DC were significantly ($p \leq 0.01$) better than FITC-bearing DC at inducing the proliferation of oxazolone-sensitised LNC at all the concentrations tested.

3.1.4.9 The effect of UVB exposure on the induction of effector cell populations in the lymph nodes

The previous experiments examined the influence of UVB exposure on the ability of DC to stimulate the proliferation of sensitised T cells. Circulating effector T cells are re-activated in the periphery by non-professional APC. In the same way sensitised T lymphocytes may have less stringent requirements for activation *in vitro*. Therefore sensitised LNC may not be the most appropriate population in which to examine UVB-induced defects in APC activity.

To examine whether UVB exposure could affect the primary induction of effector cell populations, the previous protocol was altered. Responder mice were irradiated with the standard immunosuppressive dose of UVB (1440 J/m^2 UVB, -48 and -24 hrs) or were not irradiated. UVB treated and control mice were sensitised, and, seven days later, their lymph nodes were removed and their LNC were re-stimulated *in vitro* with antigen-bearing DC.

Figure 3.1.12 shows the results from this experiment. It can be seen that FITC and oxazolone-DC induce significant ($p \leq 0.01$) antigen specific proliferative responses from FITC and oxazolone-sensitised LNC respectively, compared with the responses of the appropriate LNC population cultured alone. The proliferative

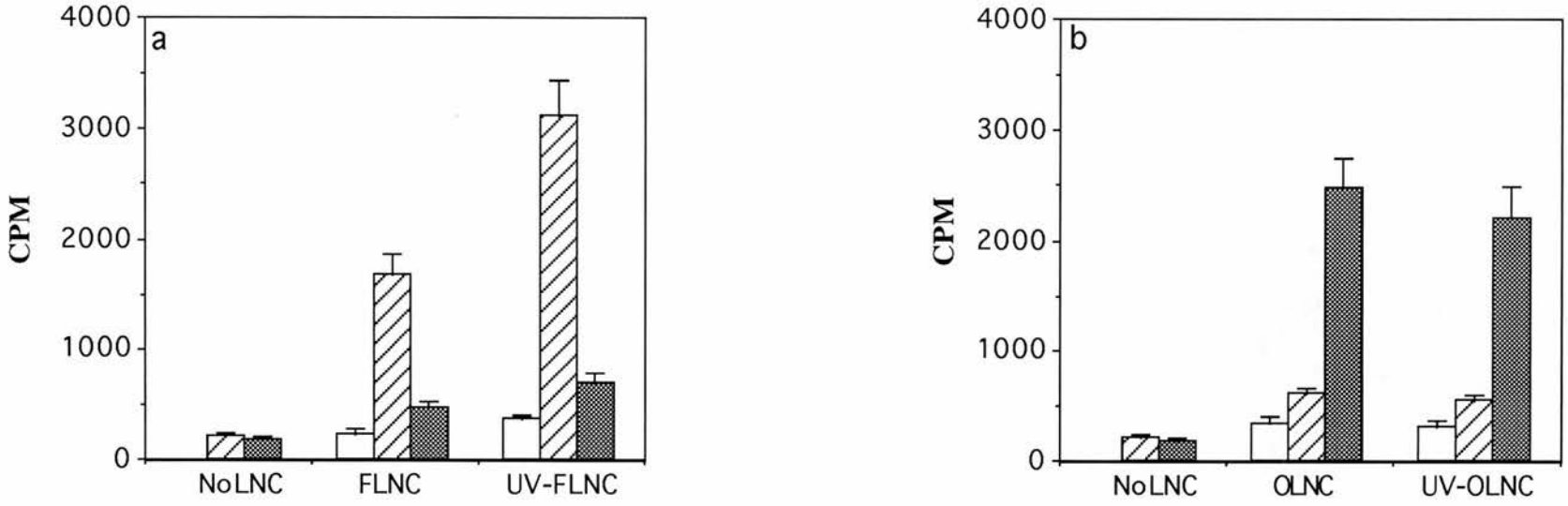

Figure 3.1.12 The effect of UVB on the induction of activated LNC populations in the DLN of FITC- (a) or oxazolone- (b) sensitised mice. Mice (n=5) were sensitised with 2.5% FITC (FLNC) or 1% oxazolone (OLNC), or were exposed to UVB (1440J/m^2, -48 and -24 hrs) prior to sensitisation with the same concentration of FITC (UV-FLNC) or oxazolone (UV-OLNC). Seven days later LNC populations were prepared from these groups and cultured alone (□), with FITC- (▨) or oxazolone- (▩) sensitised DC (from groups of 10 mice). The cells were cultured for 48 hrs and ^{3}H-methyl thymidine was added to all wells 18 hours prior to the termination of culture.

response of FITC-bearing LNC from irradiated mice cultured with FITC-bearing DC was significantly more vigorous ($p<0.01$) than the response generated when FITC-bearing LNC from unirradiated mice were cultured with the same accessory cells. There was good antigen-specificity with oxazolone-sensitised DC being considerably less able than FITC-bearing DC to stimulate proliferation of FITC-sensitised LNC. Similarly FITC-bearing DC were poor stimulators of oxazolone-sensitised LNC. The difference in proliferative activity between LNC from irradiated and non-irradiated oxazolone-sensitised mice cultured with oxazolone-sensitised DC was not significant.

Therefore, an antigen specific effector T cell population is induced in the DLN of mice sensitised via irradiated or normal skin. These LNC populations are able to proliferate in response to antigen specific stimuli. The experiment was repeated to confirm this conclusion. The data are shown in Figure 3.1.13 The protocol was the same as in Figure 3.1.12 except that the DC were used at two concentrations (1:55 and 1:100). The results confirm the findings from the previous experiment; that an antigen specific LNC population is induced in the draining lymph nodes of sensitised mice irrespective of whether they have been exposed to UVB or not. The difference in proliferative activity between LNC from irradiated and non-irradiated FITC-sensitised mice cultured with FITC-sensitised DC was not significant.

3.1.5 Effect of UVB on DC accessory function in mixed lymphocyte reactions (MLR)

3.1.5.1 Murine MLR

Initial experiments using both spleen and lymph node cell populations from C3H/HeN mice as stimulators in MLR failed. A number of variables were tested to establish whether proliferative responses could be induced. A variety of stimulator cell and responder cell concentrations were used (0.75, 1.5, 3 and $6x10^5$ cells/well).

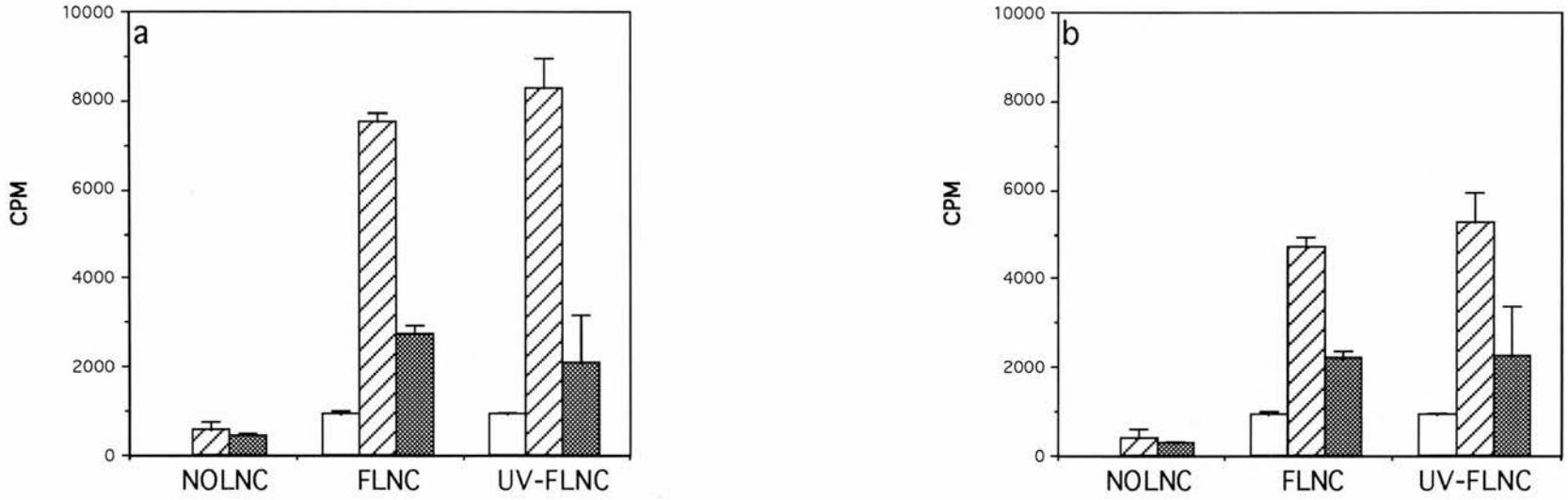

Figure 3.1.13 The effect of UVB on the induction of sensitized LNC populations in the draining lymph nodes of sensitised mice. Mice (n=5) were sensitised with 2.5% FITC (FLNC), or were exposed to UVB (1440J/m^2, -48 and -24 hrs) prior to sensitisation with the same concentration of FITC (UV-FLNC). Seven days later LNC populations were prepared from these groups and cultured alone (□), or with FITC (▨) or oxazolone (▩) bearing DC (from groups of 10 mice) at a stimulator to responder ratio of 1:55 (Figure a) or 1:100 (Figure b). The cells were cultured for 48 hrs and ^{3}H-methyl thymidine was added to all wells 18 hours prior to the termination of culture.

Timepoints from 3-6 days were tested. C3H/HeN LNC and spleen cells were used as accessory cells in MLR with LNC and spleen cells from BALB/c, C57BL/6, LTSV, 129/OLA, RBB, and transgenic mice as responders.

Because one of the defining features of DC is their ability to stimulate potently MLR, it was decided to use enriched DC populations from C3H/HeN mice as accessory cells in this assay. Initial results were disappointing with only very high concentrations of DC (1:20 stimulator:responder ratio) having any activity (see Figure 3.1.14). However, although the potency of accessory activity was low, it was specific to DC, as populations depleted of DC failed to induce proliferation at any concentration. In a previous experiment it was noted that LNC from BALB/c mice sensitised with oxazolone 48 hrs previously could be induced to proliferate by C3H/HeN DC (data not shown). It was decided therefore to examine whether sensitising BALB/c mice could increase their ability to act as responders.

3.1.5.2 The effect of antigen-priming on MLR

LNC were prepared from the auricular lymph nodes of mice sensitised with oxazolone 48 hrs earlier. In addition peripheral lymph nodes were prepared from the same mice to measure the response of non-sensitised LNC. It can be seen in Figure 3.1.15 that LNC draining oxazolone sensitised sites from BALB/c mice proliferate well in response to DC from C3H/HeN mice. In contrast LNC isolated from the peripheral lymph nodes of the same mice fail to respond under identical conditions. Therefore in all the MLR which follow, responder BALB/c mice were sensitised with 1% oxazolone 48 hrs prior to each experiment.

3.1.5.3 The effect of UVB on the stimulatory capacity of DC in antigen primed primary MLR.

When enriched DC from C3H mice were cultured with LNC from BALB/c mice sensitised previously with oxazolone, significant proliferative responses were seen using a range of stimulator to responder cell concentrations (1:15 to 1:100)

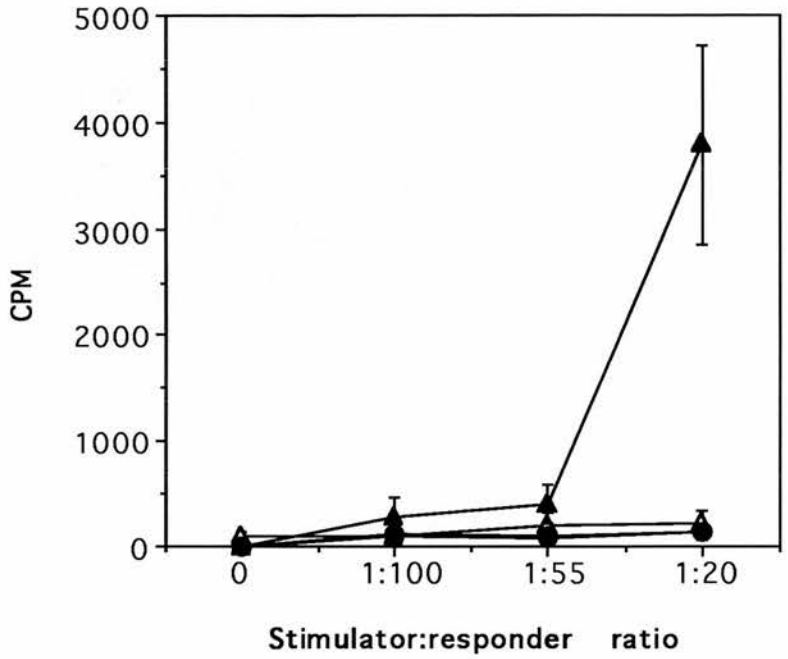

Figure 3.1.14 The ability of DC to act as allostimulatory cells *in vitro*. Responder LNC from BALB/c mice (n=3) were cultured alone or with various numbers of DC (▲) or a matching number of DC depleted cells (●) from the peripheral lymph nodes of C3H/HeN mice (n=10). Enriched (Δ) and depleted (○) DC were also cultured alone. The cells were cultured for 120 hrs and ^{3}H-methyl thymidine was added to all wells 24 hrs prior to termination of the culture. The results are expressed as mean CPM±SD for 5 replicate cultures. The proliferative CPM for BALB/c responnder LNC cultured alone was less than 100 CPM.

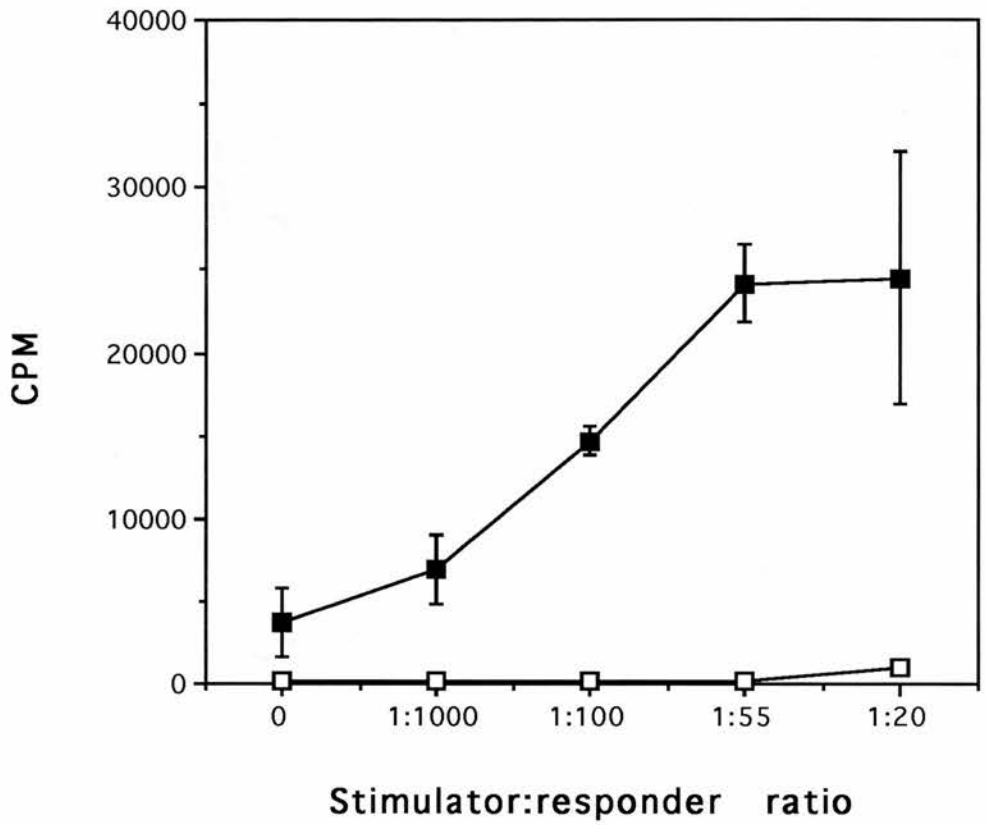

Figure 3.1.15 Responder LNC from the auricular lymph nodes (■) and peripheral lymph nodes (□) of oxazolone sensitised BALB/c mice (n=4) were cultured alone or with various numbers of DC enriched C3H/HeN mice (n=15). The cells were cultured for 120 hrs and ^{3}H-methyl thymidine was added to all wells 24 hrs prior to termination of the culture. The results are expressed as mean CPM±SD for 5 replicate cultures. The proliferative CPM for DC cultured alone were below 200 CPM.

Figure 3.1.16). The stimulation of alloresponses was dependent upon the presence of DC, as stimulator populations depleted of DC failed to induce proliferative activity. However, when DC were isolated from mice given a suppressive dose of UVB radiation, their ability to induce alloresponses was unimpaired. Even exposure of mice to a much higher dose of UVB (15 kJ/m^2) 24 hrs prior to enrichment of DC from the draining lymph nodes failed to affect the function of the DC as allostimulator cells (Figure 3.1.17).

3.1.5.4 Kinetics of antigen primed MLR

All of the antigen primed MLR were harvested on day 5 following the initiation of culture, a timepoint which is commonly used for MLR (Young and Steinman, 1988). However, because of the requirement for antigen-priming of responder LNC in this particular protocol, it was decided to look at the kinetics of this response. MLRs were prepared at a variety of stimulator:responder ratios and cultured for 3-6 days (Figure 3.1.18). It can be seen that there is a good dose response curve for the MLR. However, peak proliferation is seen at day 4 rather than day 5, although the differences in the proliferative activity on these days were not significant. Although in previous experiments UVB failed to affect the mixed lymphocyte response at a variety of stimulator:responder ratios, including those which, in this experiment, produced comparable proliferative responses at day 4 and day 5. It is possible that by missing the peak proliferative response the validity of the assay was questioned. Therefore it was decided to examine the effect of the low-dose immunosuppressive UVB protocol (1440J/m^2, -48 and -24 hrs prior to DC preparation) on the antigen primed MLR at days 4 and 5.

3.1.5.5 The effect of UVB on antigen-primed MLR at day 4 and 5.

The results from this experiment are shown in Figure 3.1.19a and b indicating the proliferative responses at day 4 and 5 respectively. In Figure 3.1.19a, the proliferative responses induced by DC from unirradiated and irradiated mice differ

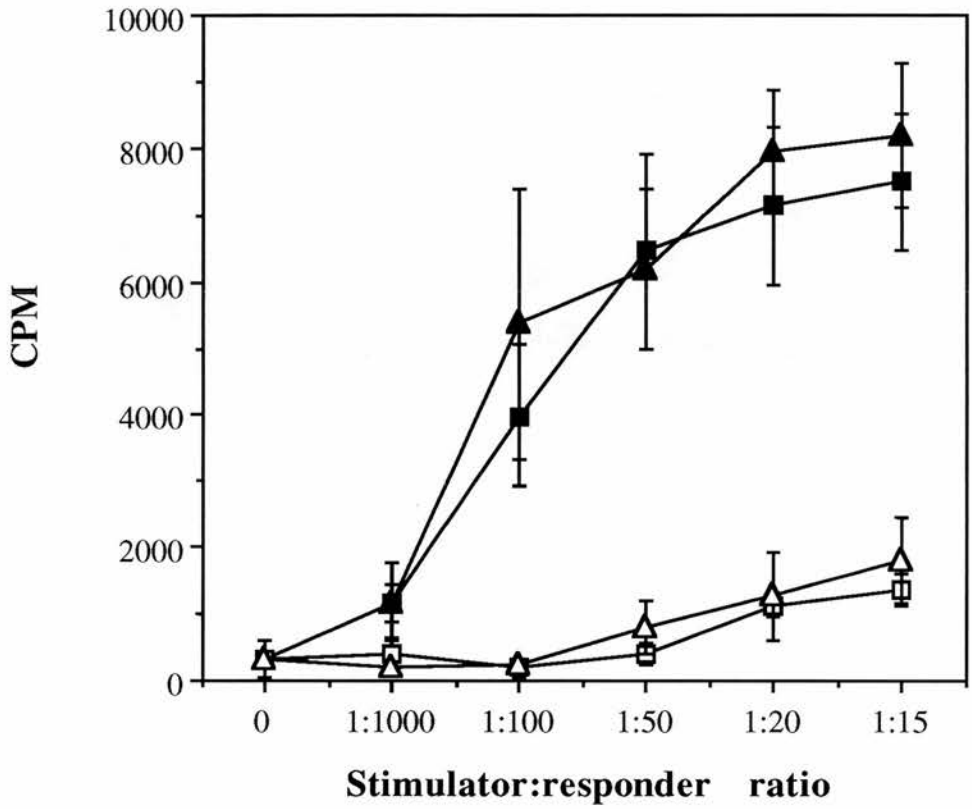

Figure 3.1.16 The effect of low dose UVB on the allostimulatory capacity of enriched DC *in vitro*. Responder LNC from oxazolone sensitised BALB/c mice (n=5) were cultured alone or with various numbers of DC from unirradiated C3H mice (n=15) (■) or UVB (1440 J/m^2 , -48 and -24 hrs) irradiated C3H mice (n=15) (▲). Responder LNC were also cultured with DC depleted populations from unirradiated (n=15) (□) and UVB irradiated mice (n=15) (△). The cells were cultured for 120 hrs and ^{3}H-methyl thymidine was added to all wells 24 hrs prior to termination of the culture. The results are expressed as mean CPM±SD for 5 replicate cultures. The proliferative CPM for DC enriched and depleted populations cultured alone were below 500 CPM.

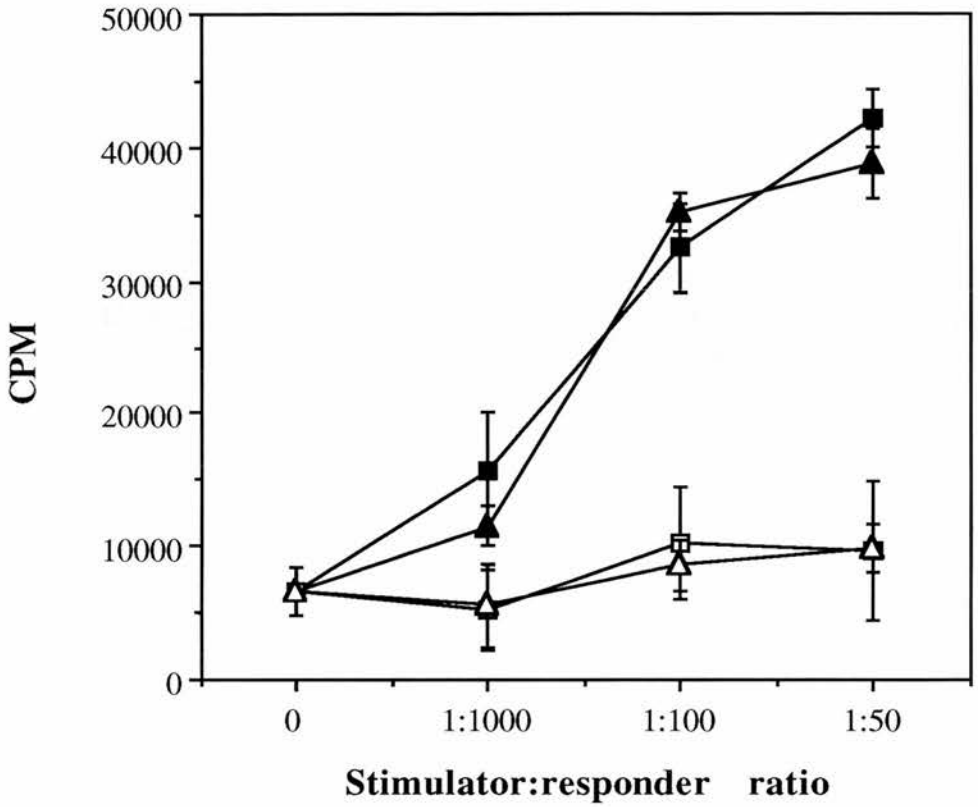

Figure 3.1.17 The effect of high dose UVB on the allostimulatory capacity of enriched DC *in vitro*. Responder LNC from oxazolone sensitised BALB/c mice (n=4) were cultured alone or with various numbers of DC from unirradiated C3H mice (n=15) (■) or UVB (15 kJ/m^2 total) irradiated C3H mice (n=15) (▲). Responder LNC were also cultured with DC depleted populations from unirradiated (n=15) (□) and UVB irradiated mice (n=15) (△). The cells were cultured for 120 hrs and ^{3}H-methyl thymidine was added to all wells 24 hrs prior to termination of the culture. The results are expressed as mean CPM±SD for 5 replicate cultures. The proliferative CPM for DC enriched and depleted populations cultured alone were below 500.

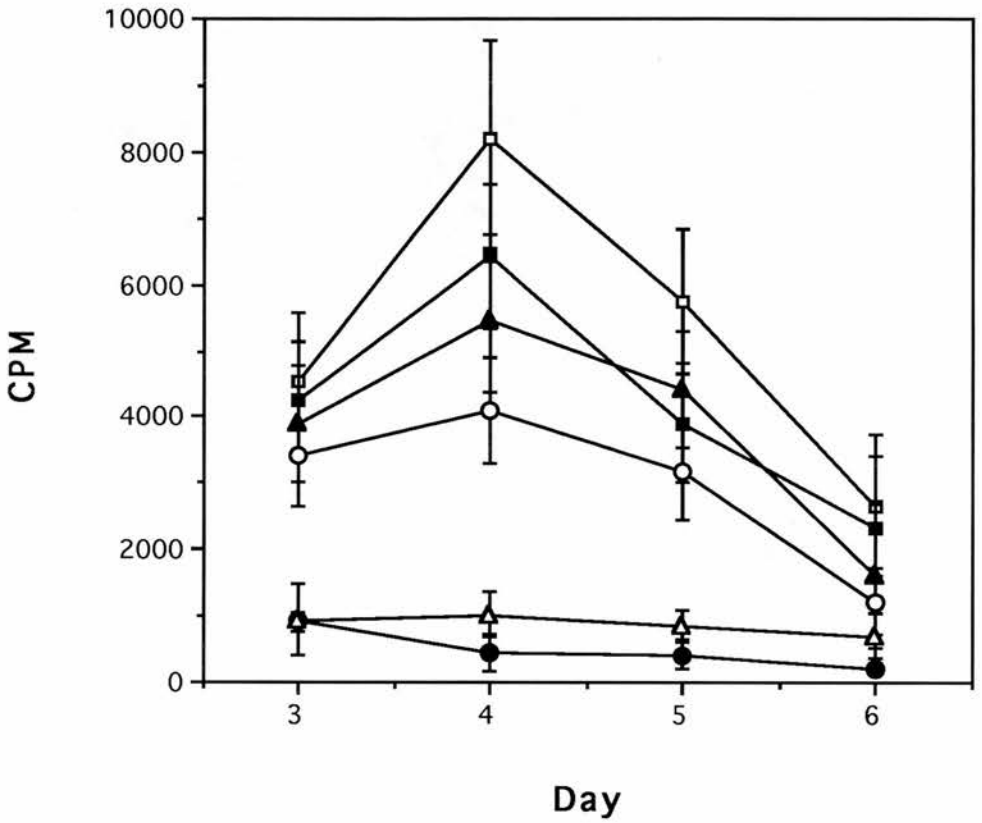

Figure 3.1.18 Kinetics of MLR. Responder BALB/c LNC (from 5 mice) were cultured alone (●) or were incubated with C3H/HeN DC (from 15 mice) at various stimulator:responder ratios; 1:20 (□), 1:40 (■), 1:55 (▲), 1:100 (○) and 1:1000 (△). The cells were cultured for 3, 4, 5 and 6 days and ^{3}H-methyl thymidine was added to all the wells 24 hrs prior to the termination of culture. The results are expressed as mean CPM±SD for 5 replicate cultures. The proliferative CPM DC cultured alone were less than 200 CPM.

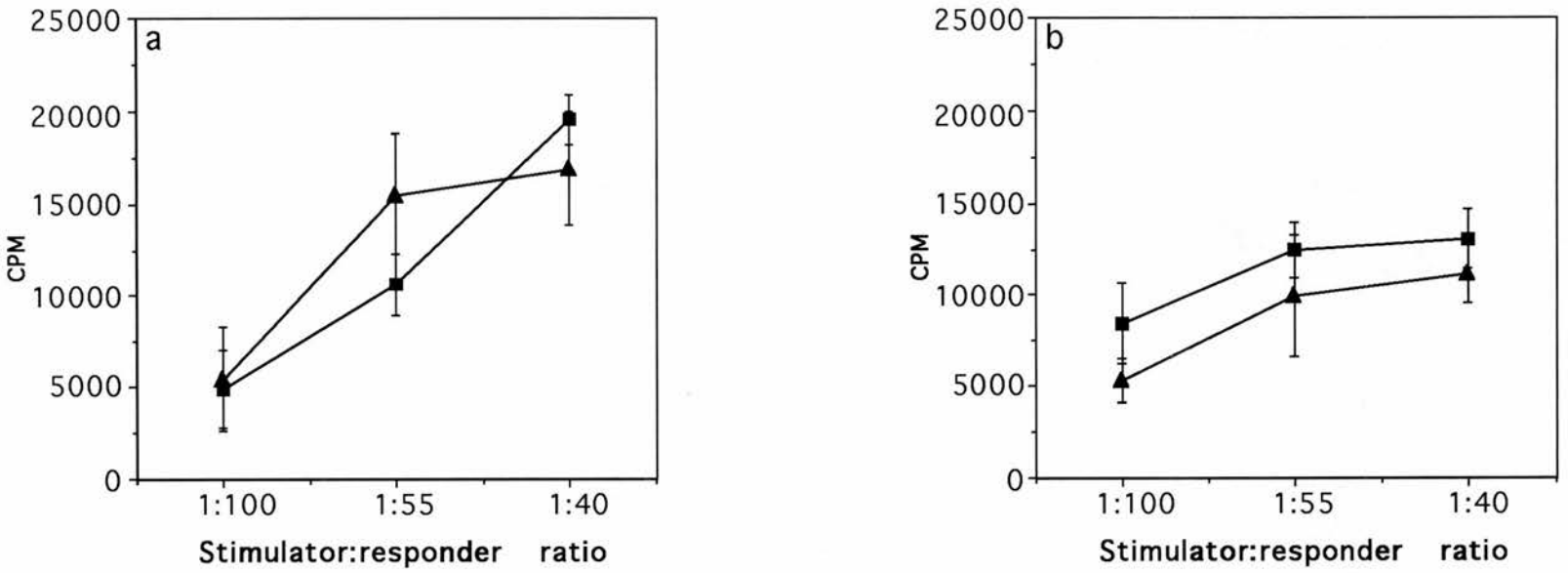

Figure 3.1.19 The effect of low dose UVB on the allostimulatory capacity of enriched DC *in vitro*. Responder LNC from oxazolone sensitised BALB/c mice (n=3) were cultured alone or with various numbers of DC enriched from unirradiated C3H mice (n=10) (■) or UVB (1440 J/m^2 , -48 and -24 hrs) irradiated C3H/HeN mice (n=10) (▲). The cells were cultured for 96 (figure a) or 120 hrs (figure b) and ^{3}H-methyl thymidine was added to all wells 24 hrs prior to termination of the culture. The results are expressed as mean CPM±SD for 5 replicate cultures. BALB/c responder LNC and DC cultured alone produced less than 950 CPM and 250 CPM respectively. These background counts were subtracted from the CPM displayed.

only at the 1:55 stimulator:responder ratio. At this ratio DC from irradiated animals induced significantly higher (p=0.025) responses. In Figure 3.1.19b, the proliferative responses were again similar at all stimulator:responder concentrations.

3.1.6 The effect of acute in vivo UVB exposure on the phenotype of lymph node DC

3.1.6.1 The characteristics of enriched DC populations

Table 3.1.1a and b show the proportion of cells within enriched DC populations that exhibits DC morphology and the percentage of cells within the large granular population (measured by flow-cytometry). There is an association between the percentage of cells with DC morphology and the proportion of cells within the DC enriched population that are large and granular, measured as a function of forward-angle and side-angle light scatter by flow-cytometry. In untreated peripheral lymph nodes (Table 3.1.1a) 53±12% of cells exhibit DC morphology by microscopy and a similar number of the enriched DC population (44±13%) were large and granular. UVB treatment failed to influence the percentage of cells with DC morphology 49±5%, or the percentage of large granular cells (38±10%) within the enriched population (Table 3.1.1b).

3.1.6.2 The characteristics of DC-depleted populations

The pellet of cells which was collected following Metrizamide centrifugation provided a DC-depleted population. Figure 3.1.20 shows the forward-angle versus side-angle light scatter plot of a DC-depleted population. DC-depletion reduces the percentage of large granular cells (region B) from around 40% to 5% . The unlabelled region contains cells with the size and granularity of lymphocytes.

3.1.6.3 The expression of ICAM-1 on DC isolated from unsensitised lymph nodes.

The effect of UVB on ICAM-1 expression on DC was examined by comparing DC enriched populations from the peripheral lymph nodes of untreated mice with DC

	a Control		b UVB	
	Percentage of cells with DC morphology	Percentage of cells that are large and granular (flow-cytometry)	Percentage of cells with DC morphology	Percentage of cells that are large and granular (flow-cytometry)
	34	57	56	49
	45	35	49	30
	60	53	48	44
	67	44	43	29
	54	53		
	57	23		
mean percentage ±SD	53±13	44±13	49±5	39±10

Table 3.1.1 The characteristics of enriched DC populations from the peripheral lymph nodes of untreated mice (n=10) (a) and the auricular lymph nodes of UVB-irradiated mice (n=10) (b). In both cases the percentage of DC within the enriched population was analyse by examining morphology using light microscopy, and size and granularity, respectively by forward and side-angle light scatter. Six separate experiments are shown in Table a and four are shown in Table b.

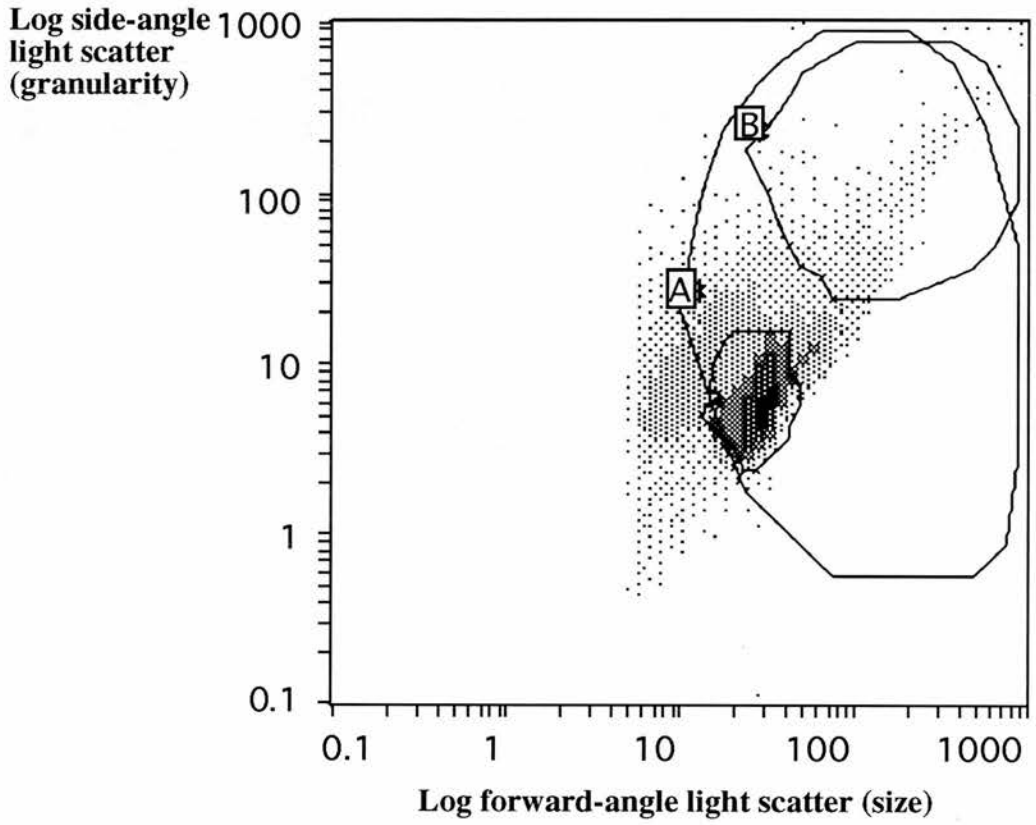

Figure 3.1.20 The size and granularity of a DC-depleted population from the peripheral lymph nodes of untreated mice (n=10). Region A was placed around the total population, and region B around the depleted large granular cell population.The unlabelled region contains cells with the size and granularity that is characteristic of lymphocytes.

populations from the auricular lymph nodes of mice exposed to UVB (1440 J/m^2, 48 and 24 hrs previously). Enriched DC populations were analysed first by their forward-angle and side-angle light scatter characteristics (Figure 3.1.21). Regions were placed around the total enriched population (excluding debris) and around the semi-discrete cell population which were large and granular (regions A and B respectively in Figure 3.1.21). Within the total enriched DC population (region A) the percentage of cells expressing ICAM-1 is raised from 37 to 55% following UVB irradiation (Figure 3.1.22). This may reflect the increased proportion of DC within enriched DC populations from irradiated animals, the percentage of large granular cells in region B (Figure 3.1.21) were raised from 34 to 58% of total cells following irradiation of mice. Similarly, ICAM-1 expression on the large granular cells (region B) is raised from 77 to 89% following irradiation (Figure 3.1.23). The intensity of ICAM-1 staining on total enriched DC populations or on the large granular cells within those populations, was not influenced by UVB (Figures 3.1.22 and 3.1.23).

Table 3.1.2 summarises the data examining the role of UVB in the alteration of ICAM-1 expression on DC. In all cases the expression of ICAM-1 by DC is unaffected or raised following UVB. In addition the intensity of ICAM-1 staining is comparable on DC from untreated and UVB irradiated mice. In three out of four experiments over 77% of DC expressed ICAM-1. In experiment 3 , where only 43-45% of DC expressed ICAM-1, there was alot of background fluorescence with the isotype control, therefore approximately half of the ICAM-1 expression was within the region set using this control. In Figure 3.1.24b, ICAM-1 expression on DC is shown by immunohistochemistry.

3.1.6.4 The effect of prior UVB prior to sensitisation with 1% oxazolone on ICAM-1 expression on DC.

In the previous experiments DC draining irradiated ears were compared with DC in pooled skin draining lymph nodes. The following experiments differ in that control mice were sensitised with oxazolone 18 hrs earlier while the other group were

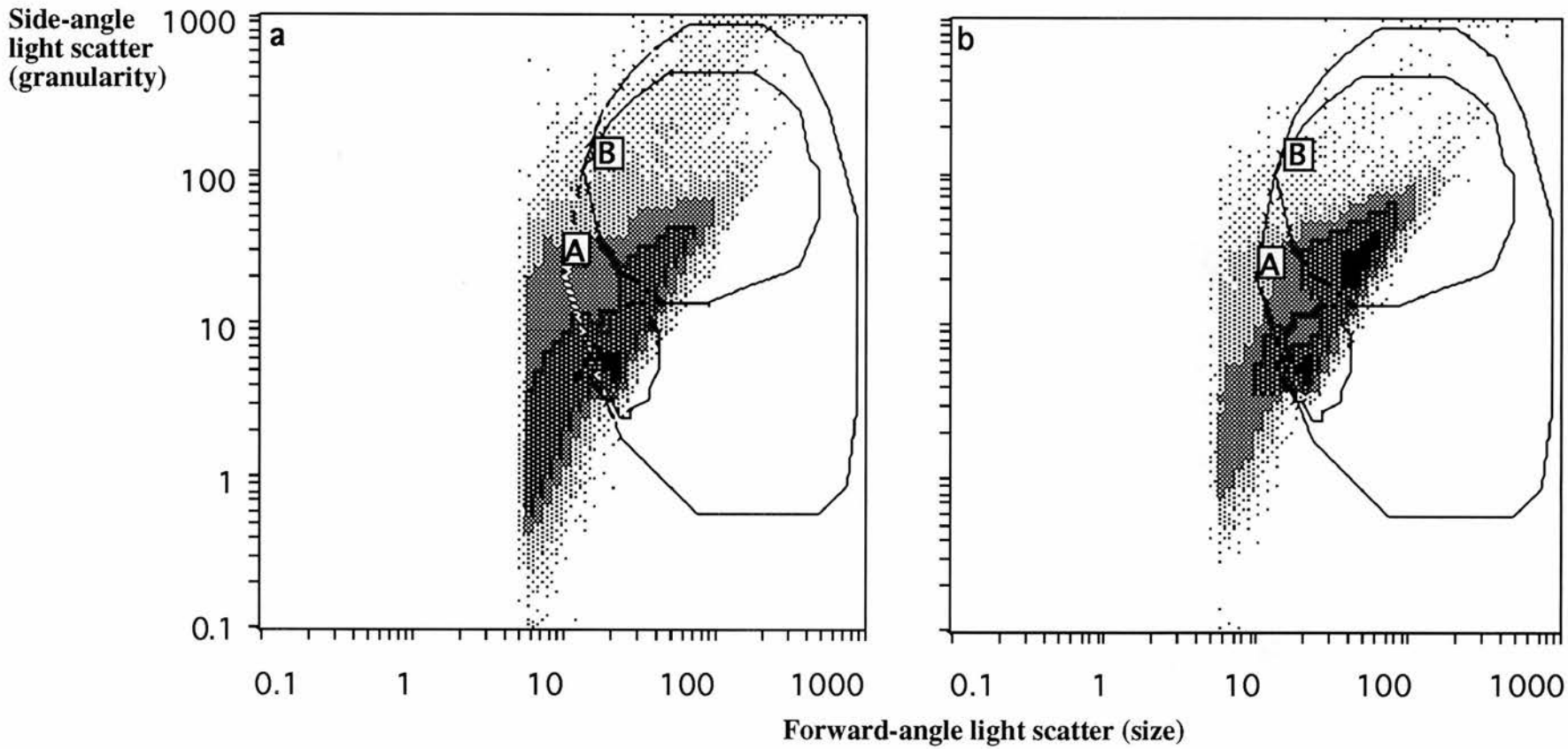

Figure 3.1.21 The size and granularity of enriched DC populations from the peripheral lymph nodes of untreated mice (n=10) in figure a, and from mice (n=10) exposed to UVB (1440 J/m^2, -48 and -24 hrs) in figure b. Region A was placed around the total population, and region B around the semi-discrete large granular cell population.

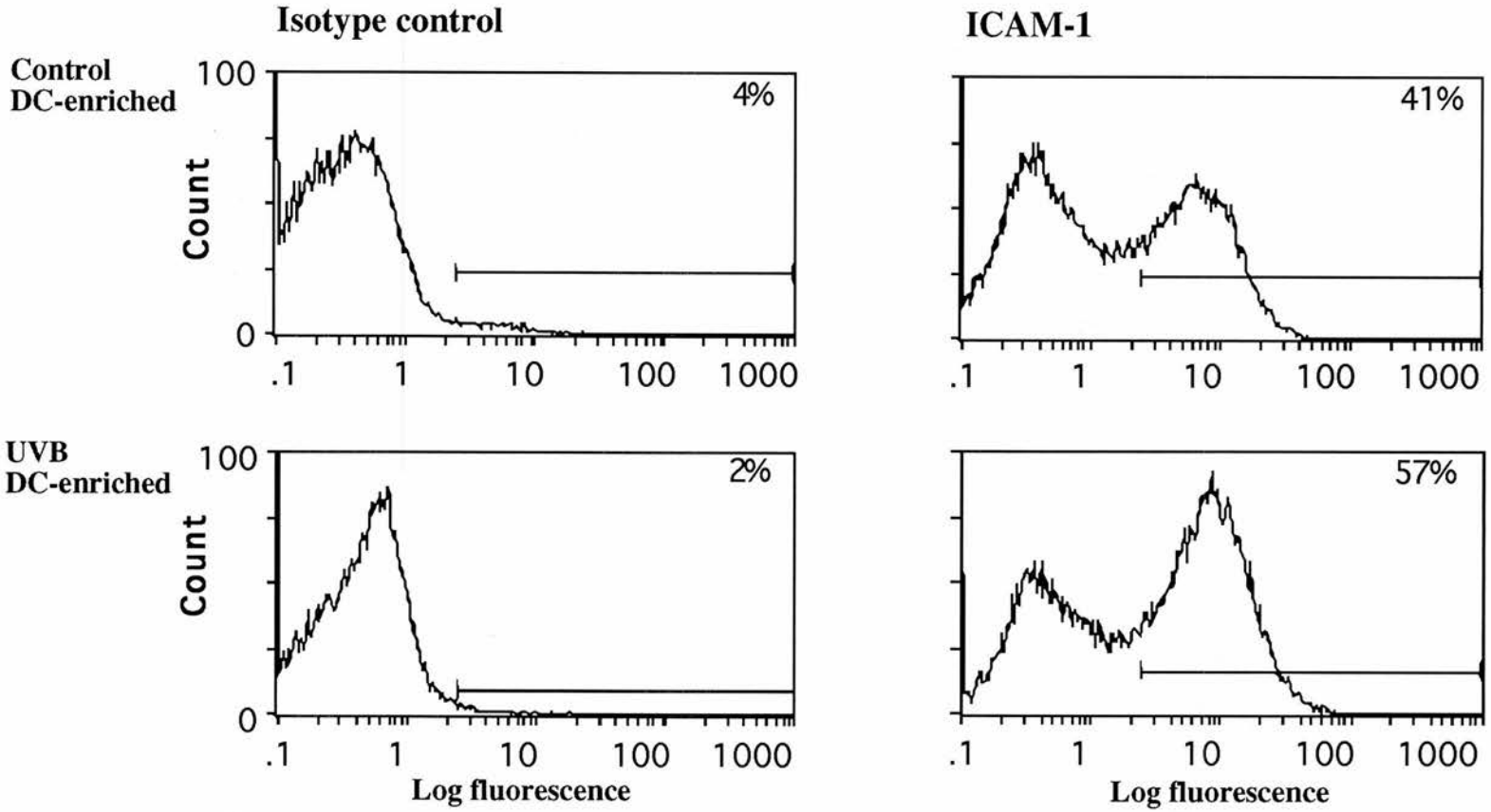

Figure 3.1.22 ICAM-1 expression on total DC-enriched cells from the peripheral lymph nodes of unirradiated mice (n=10) and UVB irradiated mice (n=10). The line on each graph represents the region within which fluorescence is considered positive, these regions were set using the isotype controls. The percentage of positive cells in this region is stated on each histogram.

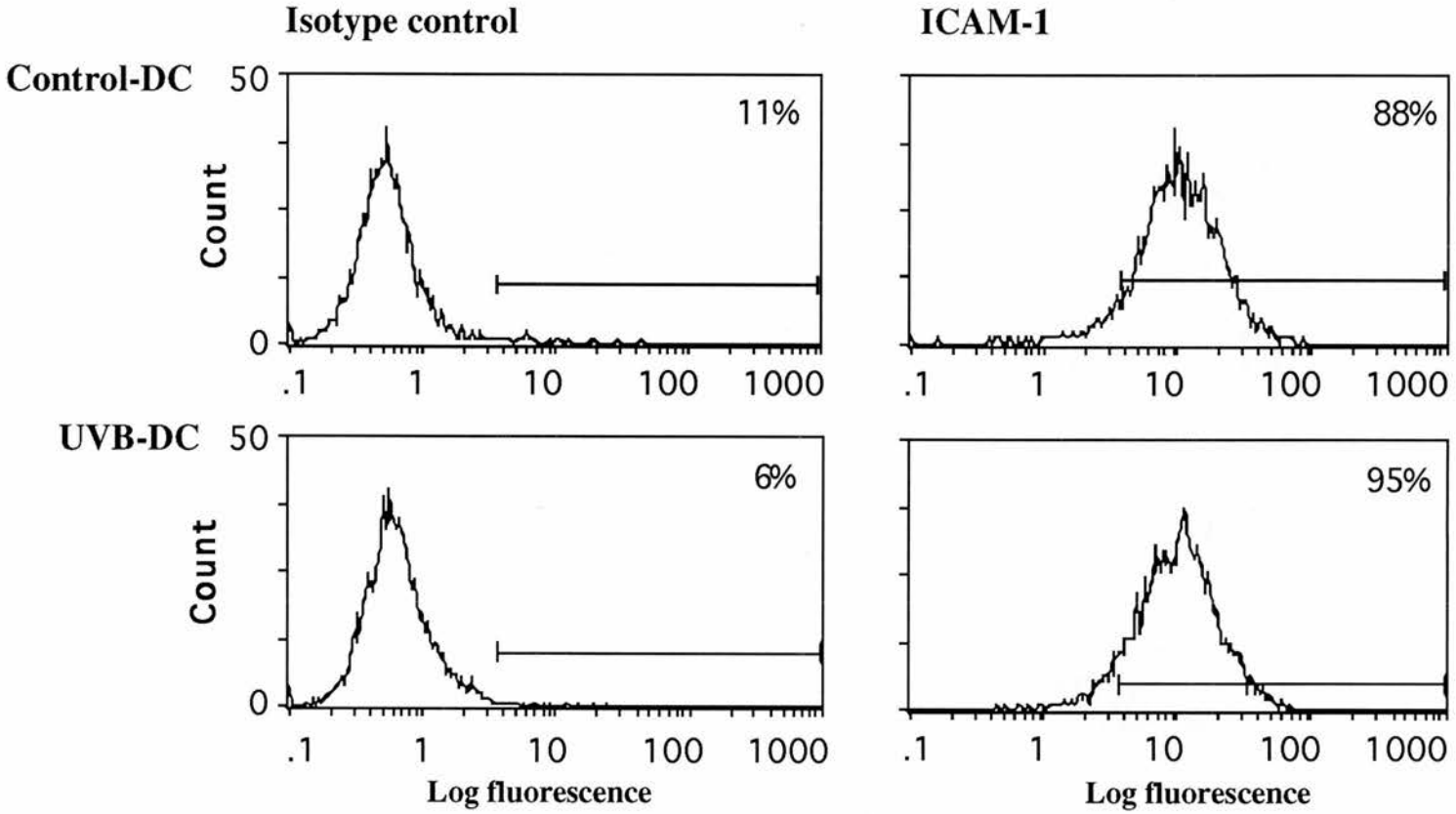

Figure 3.1.23 ICAM-1 expression on DC from the peripheral lymph nodes of untreated mice (n=10) (control) or from mice (n=10) exposed to UVB (1440 J/m^2, -48 and -24 hrs). The line on each graph represents the region within which fluorescence is considered positive, these regions were set using the isotype controls. The percentage of positive cells in this region is stated on each histogram.

	Control	UVB
Experiment	%	%
1	93	96
2	94	96
3	43	45
4	77	89

Table 3.1.2 The effect of UVB on the percentage of DC expressing ICAM-1. DC were enriched by density gradient centrifugation from the peripheral lymph nodes of untreated mice (n=10) or from the auricular lymph nodes of C3H/HeN mice (n=10) exposed to UVB (1440 J/m^2, -48 and -24 hours). ICAM-1 expression was analyzed by indirect fluorescent labelling and flow-cytometry. The percentage of cells expressing ICAM-1 (%) is shown, the results have been adjusted for background fluorescence by subtracting the isotype control value in each experiment.

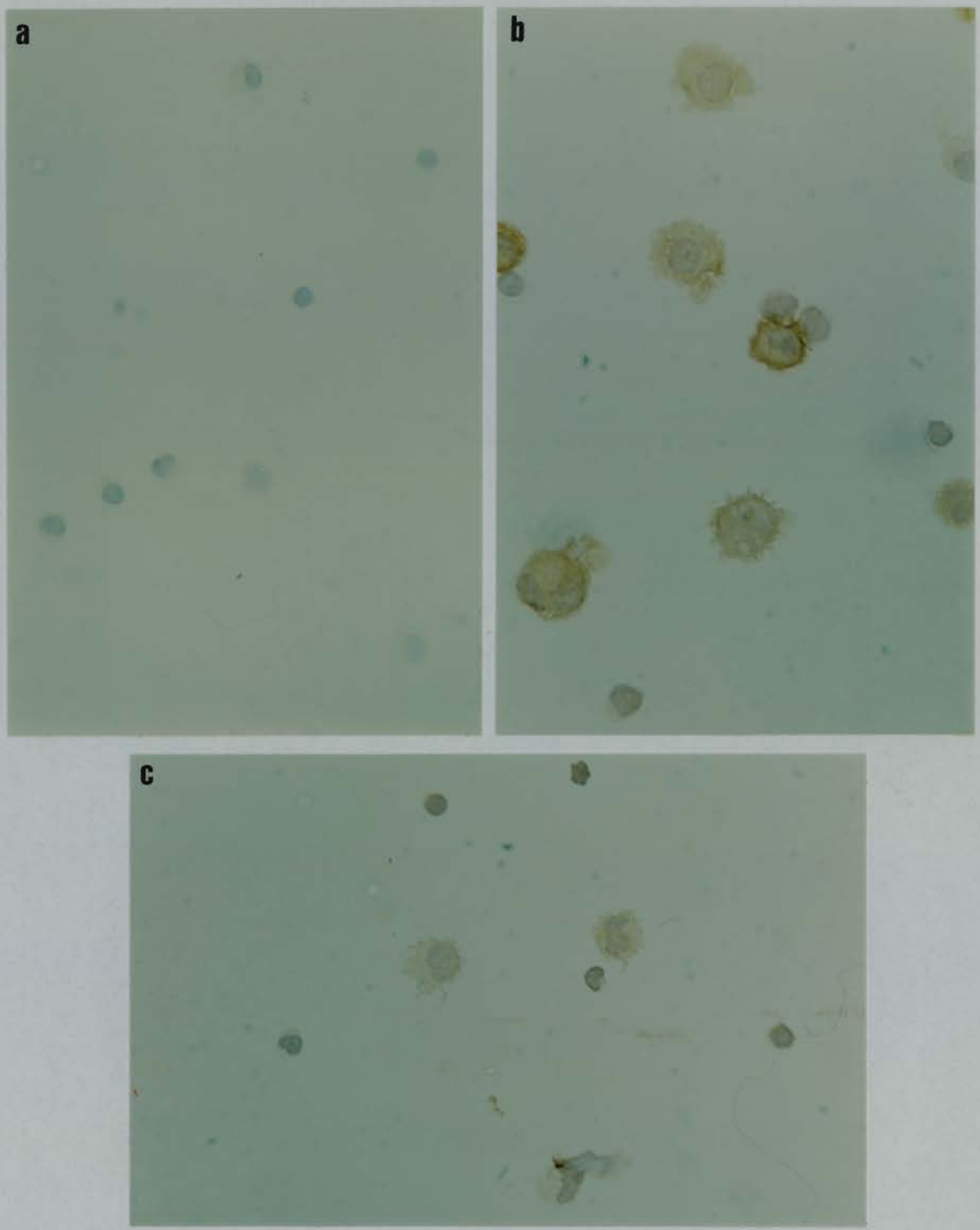

Figure 3.1.24 The expression of ICAM-1 and MHC class II on enriched DC populations. DC were enriched by Metrizamide centrifugation of LNC from the auricular lymph nodes of mice 18 hrs following oxazolone sensitisation. Cytospins were prepared and the expression of dinitrophenol (isotype control for ICAM-1) (a), murine ICAM-1 (b) or murine Ia was examined (c), using an immunoperoxidase method. Staining with the isotype control for MHC class II gave similar results as seen in Figure 3.1.24a. Magnification x25 (a and c), x40 (b).

exposed to UVB (1440 J/m^2, -48 and -24 hrs) prior to ear painting with oxazolone. Enriched DC populations were prepared from the auricular lymph nodes of each group and ICAM-1 expression was examined. Table 3.1.3 shows the results from these experiments. In experiments 5 and 7 it can be seen that UVB exposure has little effect on the percentage of DC expressing ICAM-1. Indeed, the mean percentage of DC that express ICAM-1 was 89% in the control group and 92% in the irradiated group. Similarly there was no significant change in the intensity of ICAM-1 expression (data not shown)

3.1.6.5 The effect of UVB on the expression of MHC class II on DC isolated from unsensitised and oxazolone sensitised lymph nodes.

It can be seen in Table 3.1.4 that in experiments 12 and 13 that UVB failed to affect significantly the expression of MHC class II on DC. Like ICAM-1 expression MHC class II expression is variable, but the variability tended to arise from background staining using the isotype controls. The very low level of MHC class II in experiment 14 for example, reflects high background staining (histogram not shown). The histograms from experiment 12 are presented in Figure 3.1.25. It can be seen from the histograms that the intensity of MHC class II expression on DC was similar on DC from unirradiated and UVB irradiated mice. This result was reproduced in experiment 13 in Table 3.1.4 (data not shown). Looking at the enriched population as a whole, UVB exposure causes the percentage of MHC Class II$^+$ cells to rise from 30-46% (data from experiment 12). In Figures 3.1.24a and c respectively, the expression of human MHC class I (isotype control) and murine MHC class II on DC is shown by immunohistochemistry.

	Control	UVB
Experiment	%	%
5	94	94
6	ND	96
7	90	86
8	94	ND
9	79	ND

Table 3.1.3 DC were enriched from auricular lymph nodes 18 hours following oxazolone sensitisation of unirradiated C3H/HeN mice (n=10) or from mice (n=10) exposed to UVB (1440 J/m2, -48 and -24 hours) prior to sensitisation. The percentage of cells expressing ICAM-1 (%) is shown, the results have been adjusted for background fluorescence by subtracting the isotype control value in each experiment. ND=not done.

a

	Control	UVB
Experiment	%	%
10	87	ND
11	ND	80
12	75	80

b

	Control	UVB
Experiment	%	%
13	71	66
14	56	ND

Table 3.1.4 The effect of UVB on the percentage of MHC class II expression on DC. In Table a, DC were enriched by density gradient centrifugation from the peripheral lymph nodes of untreated mice (n=10) or from the auricular lymph nodes of C3H/HeN mice (n=15) exposed to UVB (1440 J/m^2, -48 and -24 hours). In Table b, DC were enriched from auricular lymph nodes 18 hours following oxzazolone sensitisation of unirradiated C3H/HeN mice or from mice exposed to UVB (1440 J/m^2, -48 and -24 hours) prior to sensitisation. Cells were stained for MHC class II expression and analysed by flow-cytometry. The percentage of cells expressing MHC class II (%) is shown, the results have been adjusted for background fluorescence by subtracting the isotype control value from each experiment.

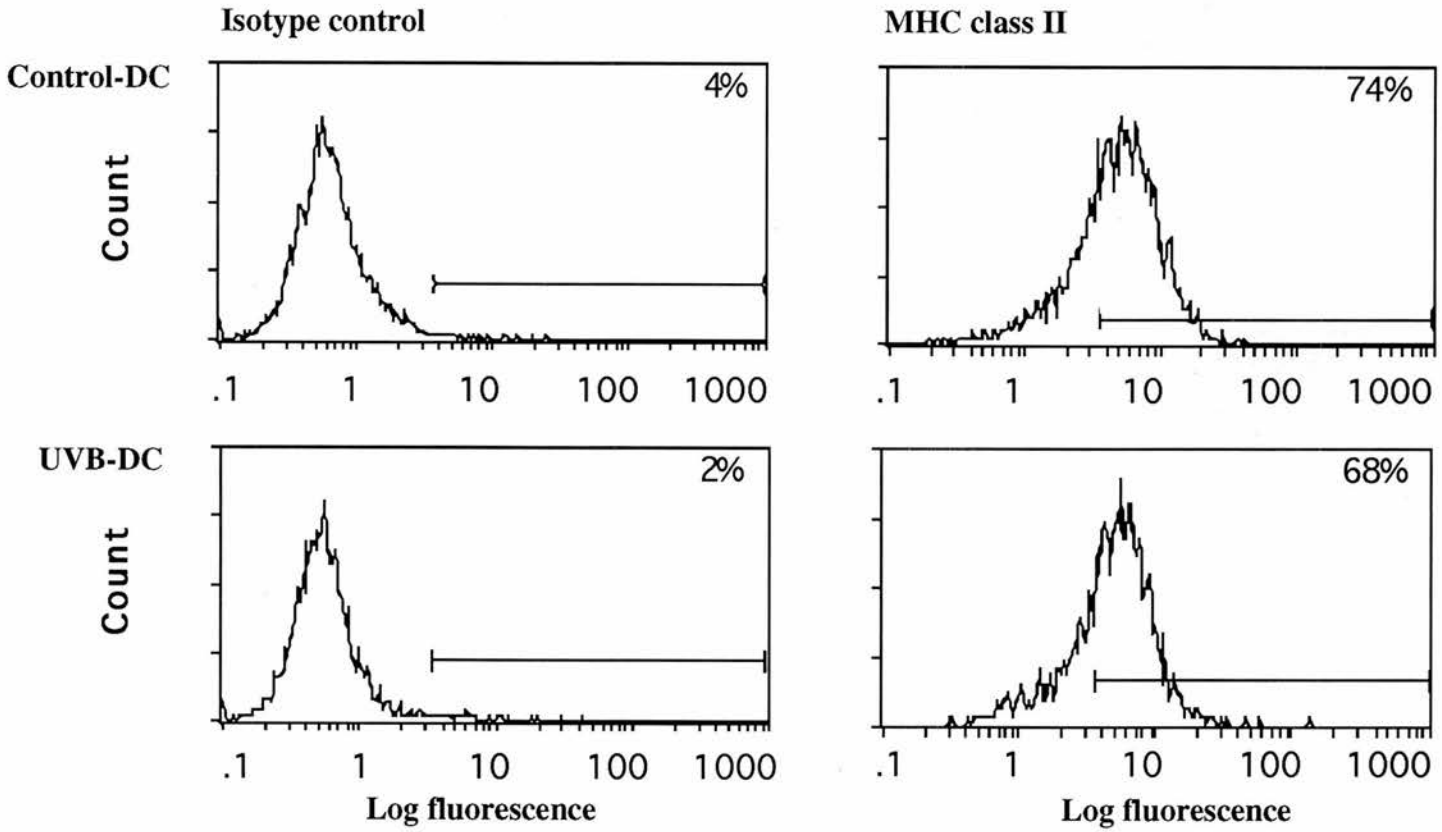

Figure 3.1.25 MHC class II expression on DC from peripheral lymph nodes of untreated mice (n=10) (control-DC), or from mice (n=15) exposed to UVB (1440 J/m^2, -48 and -24 hours). The line on each graph represents the region within which fluorescence is considered positive, these regions were set using the isotype controls. The percentage of positive cells in this region is stated on each histogram.

3.1.6.6 The effect of UVB on the expression of B7-2 (CD86) on DC isolated from oxazolone sensitised lymph nodes.

Table 3.1.5 shows the results from experiments examining the effect of prior UVB exposure on DC expression of B7-2 in lymph nodes draining an oxazolone sensitised site. In no experiments does UVB cause a meaningful reduction in the percentage of cells expressing B7-2. The mean percentage expression (ignoring the low value in experiment 15) was 58±16 and 57±13% for the control and UVB treated groups respectively. While 80-90% of DC express ICAM-1 and MHC class II, only around 60% of DC express B7-2. Again there are large inter-experimental difference in the % of DC expressing B7-2, as previously this reflected the background fluorescence which tended to vary between experiments. The histograms from experiment 16 are shown (Figure 3.1.26). It can be seen in the histograms that the intensity of B7-2 expression on DC was not altered by UVB exposure, this was similar in all experiments (experiments 15, 17 in Table 3.1.5).

3.1.6.7 Summary

In Figure 3.1.27 the histograms showing ICAM-1 (experiment 4), MHC class II (experiment 12) and B7-2 (experiment 16) expression were overlaid in order to emphasise the similarity in the expression of these molecules on DC taken from untreated and UVB irradiated mice.

3.1.7 Overall summary of the effect of UVB on DC function and phenotype

An acute UVB exposure protocol was used to examine the effect of UVB on DC *in vivo*. Mice were irradiated with an immunosuppressive dose of UVB, and the function and phenotype of DC accumulating in the DLN was examined. Exposure to UVB prior to sensitisation did not reduce the ability of DC to induce proliferative responses of hapten sensitised LNC. The accessory function of DC in MLR was also

unaffected by prior exposure to an immunosuppressive dose of UVB. The expression of MHC class II, ICAM-1 and B7-2 (CD86) on DC was also examined. UVB failed to affect the percentage of DC expressing these markers or the surface density of their expression. Therefore, in the absence of study limitations (discussed in section 4.1.5), these results provide no evidence to support the view that the immunosuppression following low-dose UVB exposure results from direct UVB-induced changes in DC. This contradicts *in vitro* studies using LC isolated from the skin of mice and humans (Tang and Udey, 1991; Rattis *et al.* 1995). In those studies, low-doses of UVB inhibited the culture-induced differentiation of LC into cells with the accessory function and phenotype associated with lymph node DC. However, since the *in vitro* and *in vivo* systems differ so markedly, it is possible that the results gained in the *in vitro* system reflect cellular responses to cytotoxic doses of UVB (Tang and Udey, 1992).

	Control	UVB
Experiment	%	%
15	28	53
16	74	72
17	42	46
18	58	ND

Table 3.1.5 The effect of UVB on B7-2 expression on DC isoloated from sensitised lymph nodes. DC were enriched from auricular lymph nodes 18 hours following oxazolone sensitisation of unirradiated C3H/HeN mice (n=10) or from mice exposed to UVB (1440 J/m2, -48 and -24 hours) prior to sensitisation (n=10). The percentage of cells expressing B7-2 (%) is shown, the results have been adjusted for background fluorescence by subtracting the isotype control from each experiment. ND=not done.

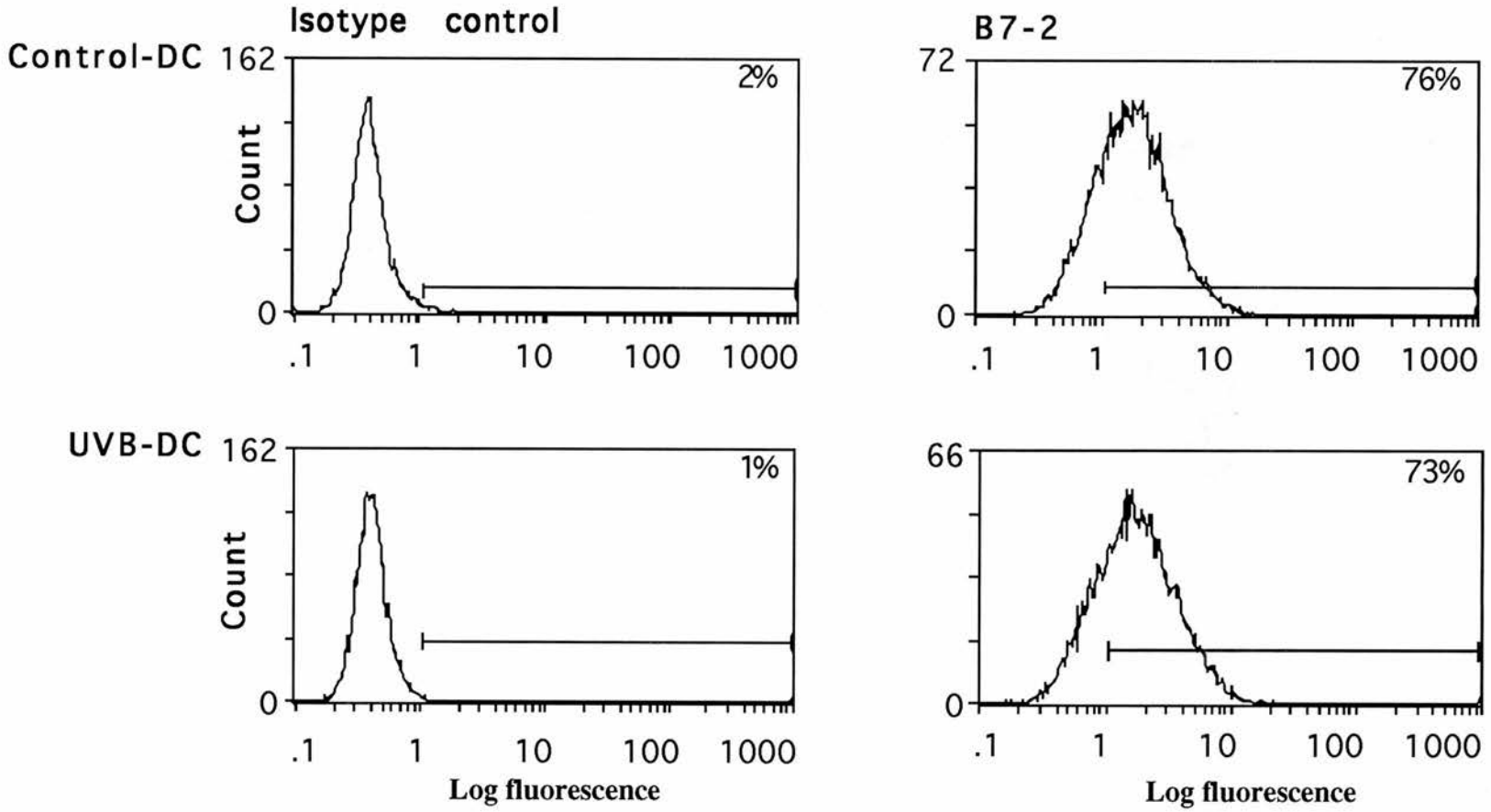

Figure 3.1.26 B7-2 expression on DC from the auricular lymph nodes of oxazolone sensitised mice (n=10) (control-DC) or from mice (n=10) exposed to UVB (1440 J/m^2, -48 and -24 hrs) prior to sensitisation. The line on each graph represents the region within which fluorescence is considered positive, these regions were set using the isotype controls. The percentage of positive cells in this region is stated on each histogram.

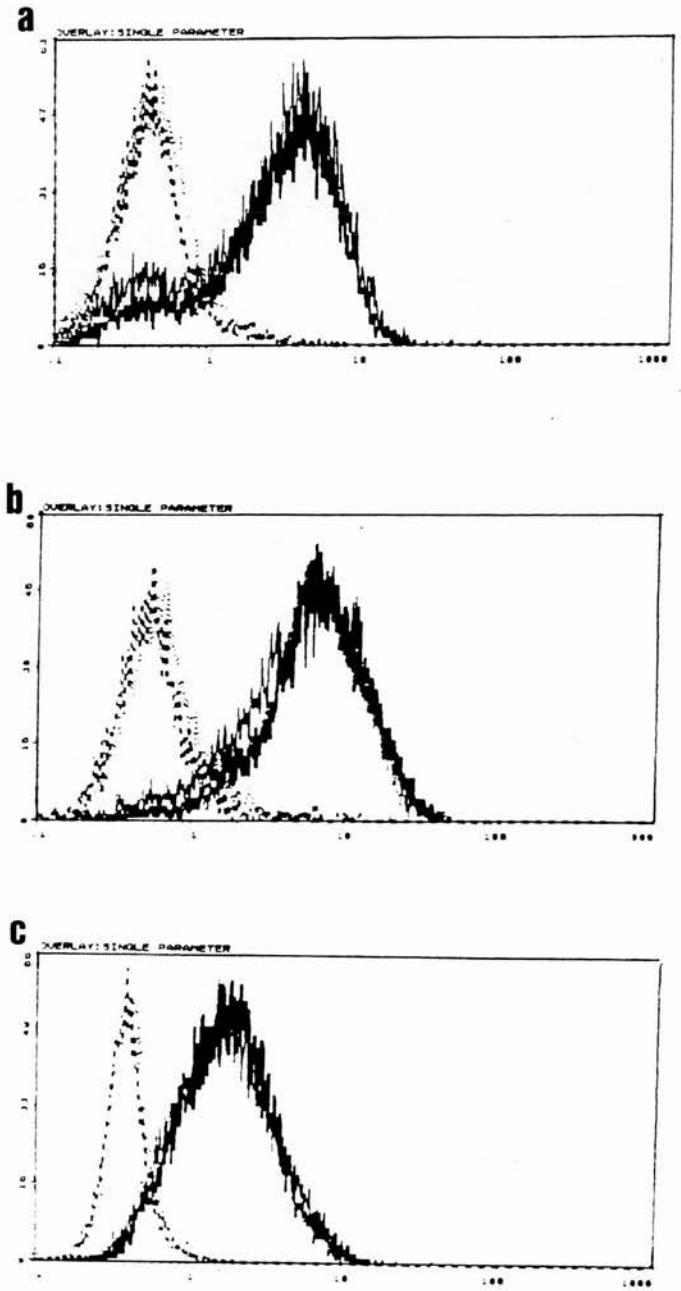

Figure 3.1.27 The effect of local UVB exposure on the phenotype of DC enriched from the draining lymph node. Mice were unirradiated or exposed to UVB (2880 J/m^2 total) prior to ear painting with oxazolone. DC were enriched from DLN 18 hrs later. The phenotype of the DC was analysed using flow cytometric analysis. Ia (a), ICAM-1 (b) and B7-2 (c) expression on DC from control (thick solid line) and UVB irradiated mice (thin solid line) was examined. The appropriate isotype controls were included for control DC (thick hatched line) and UVB DC (dotted line).

3.2 The production of cytokines in the lymph nodes and skin of mice during the induction phase of contact sensitisation

3.2.1 Introduction

Interleukin-6 (IL-6) is a cytokine produced by a wide variety of cells in response to injury, infection and a range of other cytokines. It acts on a number of cell populations and tissues, with its main effects being the stimulation of growth and differentiation of B and T lymphocytes, the initiation of acute phase protein production by hepatocytes and the induction of responsiveness to IL-3 in bone marrow stem cells (Van Snick, 1990). The production of IL-6 in DLN following contact sensitisation may be important in the induction of effector T cell populations. This hypothesis is supported by three observations. Firstly, that the induction phase of skin sensitisation in BALB/c mice is characterised by the stimulation of proliferative responses in lymph nodes draining the site of exposure (Scholes *et al.* 1992) and that this proliferative response is associated with the production of IL-6 (Hope *et al.*1994). Secondly, that the predominant source of IL-6 within LNC is the immunostimulatory DC population that accumulates in the DLN following sensitisation (Hope *et al.*1995; Cumberbatch *et al.*1996). Thirdly, that IL-6 is important in the early stages of T cell activation, particularly in the induction of IL-2 responsiveness in T lymphocytes (Van Snick, 1990).

UVB-induced immunosuppression may reflect the induction of altered effector cell populations in lymph nodes following UVB exposure. In mice irradiation prior to infection with HSV induced the appearance of a T cell population in the spleen which could transfer suppression of DTH responses (Howie *et al.*1986). Since IL-6 is important in the early stages of effector T cell activation, the purpose of these investigations was to investigate the production of IL-6 in two strains of mice which show differing susceptibility to the immunosuppressive effects of UVB, namely C3H/HeN which are UVB susceptible and BALB/c which are UVB resistant (Streilein and Bergstresser, 1988; Noonan and Hoffman, 1994).

3.2.2 Suppression of CH responses by low-dose UVB

C3H/HeN and BALB/c mice have been characterised as being, respectively, UVB susceptible and UVB resistant to low-dose local UVB-induced immunosuppression (Streilein and Bergstresser, 1988) and to systemic UVB-induced inmmunosuppression (Noonan and Hoffman, 1994) over a range of UVB doses. To confirm that these mice display consistent differences in susceptibility to UVB, the shaved backs of mice were exposed to 2x1440 J/m^2 of broadband UVB 48 and 24 hrs prior to sensitisation on the irradiated site with oxazolone. The results for C3H/HeN mice have been shown in an earlier section (Figures 3.1.1 and 3.1.2), but will be reproduced here for convenience. Figure 3.2.1 shows the suppression of CH reactions in C3H/HeN but not BALB/c mice following exposure to UVB. CH responses were suppressed by around 31% ($p=0.05$) in C3H/HeN mice, while in BALB/c mice which showed comparable CH responses, UVB-exposure prior to sensitisation failed to suppress the CH response. In the first experiment, BALB/c mice were exposed to UVB without ear protection, it was noted that BALB/c mice, unlike C3H/HeN mice, showed UVB-induced damage (erythema) to their ears by day 6 following sensitisation. This damage may be reflected in the large SEM seen in the UVB irradiated BALB/c mice in Figure 3.2.1. Because of this it was decided to anaesthetise mice and cover their ears during the irradiation. The results from this experiment are shown in Figure 3.2.2. In this experiment the ear swelling of C3H/HeN mice was comparable to that seen in the previous experiment (Figure 3.2.1), however in BALB/c mice the swelling responses were much lower, but again UVB failed to cause a suppression of the CH response.

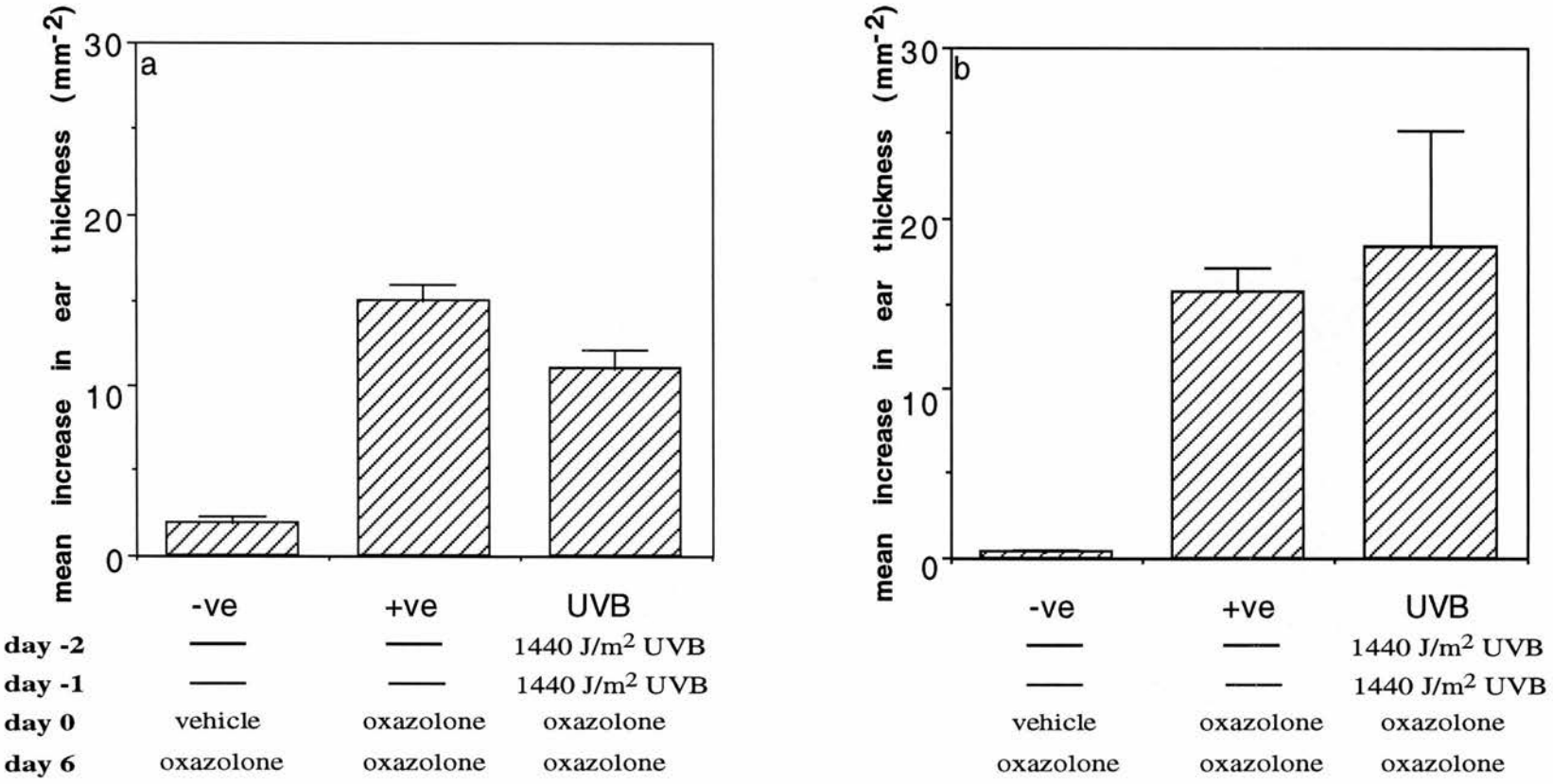

Figure 3. 2. 1 Suppression of CH responses to oxazolone by UVB in C3H/HeN (a) and BALB/c mice (b). Mice were exposed to UVB (1440 J/m^2, -48 and-24 hrs) or were unirradiated, prior to epicutaneous application of vehicle (AOO) or oxazolone (Ox) on the same site. Six days later the ears of all mice were challenged with oxazolone and the results expressed as the mean 24 hour increase in ear swelling in mm-2± SEM, n=7.

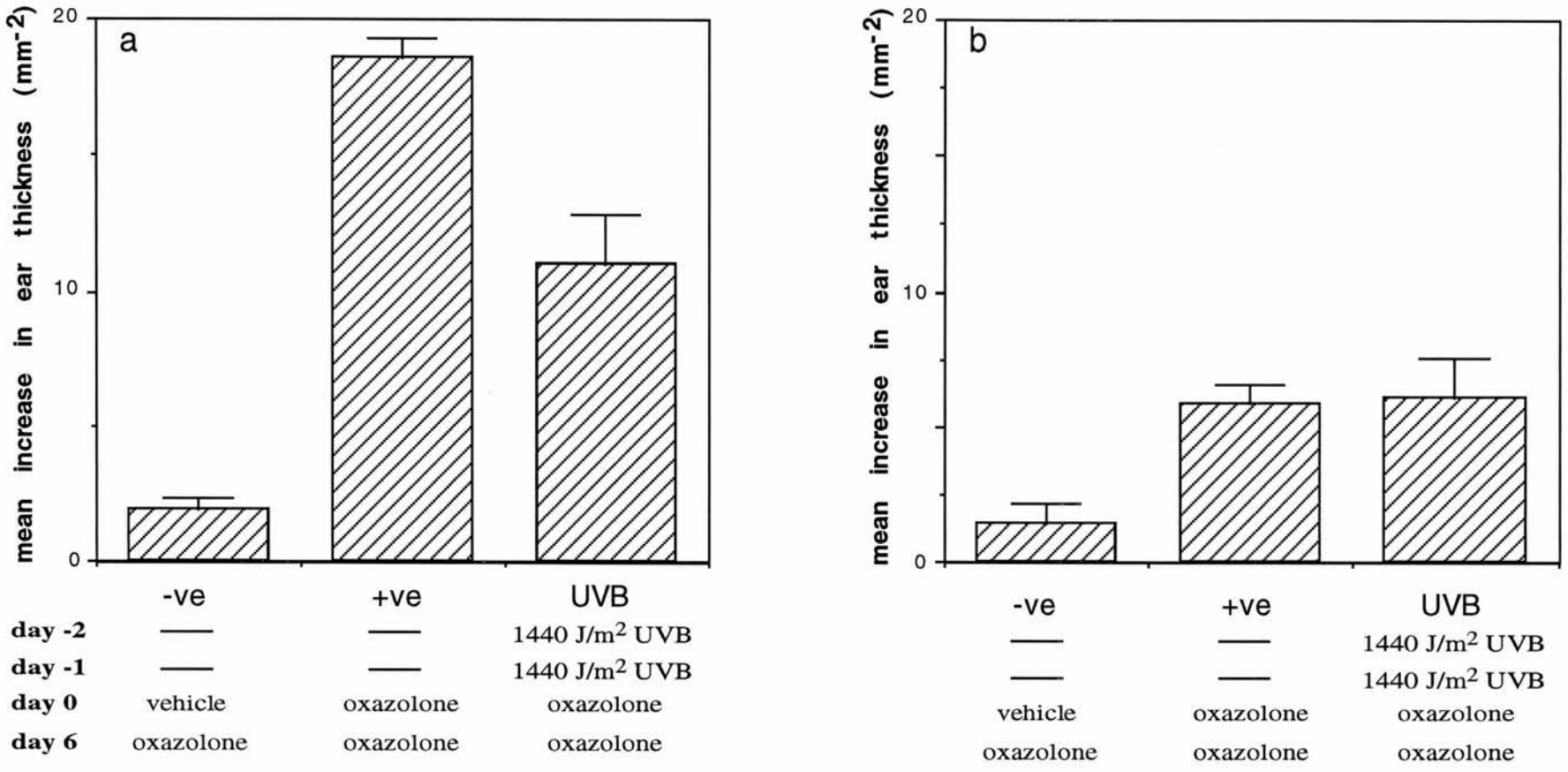

Figure 3.2.2. Suppression of CH response to oxazolone by UVB in C3H/HeN (Figure a) and BALB/c mice (Figure b). Mice were exposed to UVB (2x1440 J/m^2) or were unirradiated (-ve and +ve) prior to sensitisation with oxazolone (+ and UVB) or vehicle on the irradiated site. During the irradiation the ears of mice were protected from UVB. Six days later the ears of all mice were challenged with oxazolone and the results expressed as the mean increase in ear swelling in mm^{-2}± SEM.

3.2.3 *Proliferation and IL-6 production by LNC* in vitro

In both C3H/HeN and BALB/c strains, the kinetics of oxazolone induced proliferation were similar, peaking at 2-3 days after sensitisation and decreasing by 5 days (Figure 3.2.3 a and b). Peak responses were around 65000 CPM for C3H/HeN mice and 38000 CPM for BALB/c mice. Despite exhibiting vigorous proliferation, LNC from C3H/HeN mice produced very small amounts of IL-6; 0.3-0.4 ng/ml on days 2 and 3, and below the limits of detection (<0.15 ng/ml) by day 5 after sensitisation (Figure 3.2.3 a). In contrast, while LNC from BALB/c mice produced low levels of IL-6, 0.3 and <0.15 ng/ml on day 2 and 5 respectively, they produced comparatively large amounts of IL-6 (8-10 ng/ml) on day 3 after ear painting with oxazolone (Figure 3.2.3b). Proliferative responses were below 5000 CPM and the concentration of IL-6 in supernatants was below detectable levels (0.15 ng/ml) at all times following vehicle treatment (data not shown).

This experiment was performed with similar results on four separate occasions. The data for the day 3 timepoint are presented in Table 3.2.1. It can be seen that the LNC proliferative responses induced by oxazolone were highly variable in both strains (20000-70000 CPM). However the mean proliferative responses for both strains were around 40000 CPM and there was no significant inter-strain difference. There was considerably less variability in cytokine levels, with C3H/HeN mice producing low to undetectable levels and BALB/c mice producing significantly ($p \leq 0.025$) higher amounts of IL-6 in each of the 4 independent experiments (around 35-40 fold higher). Similar levels of IL-6 production following oxazolone sensitisation of BALB/c mice have been reported previously (Hope et al. 1994).

To ascertain whether the level of IL-6 produced by LNC following sensitisation with oxazolone could be correlated with the responsiveness of strains to the immunosuppressive effects of UVB, the proliferative responses and IL-6 production by LNC from further UVB susceptible and resistant strains was characterised.

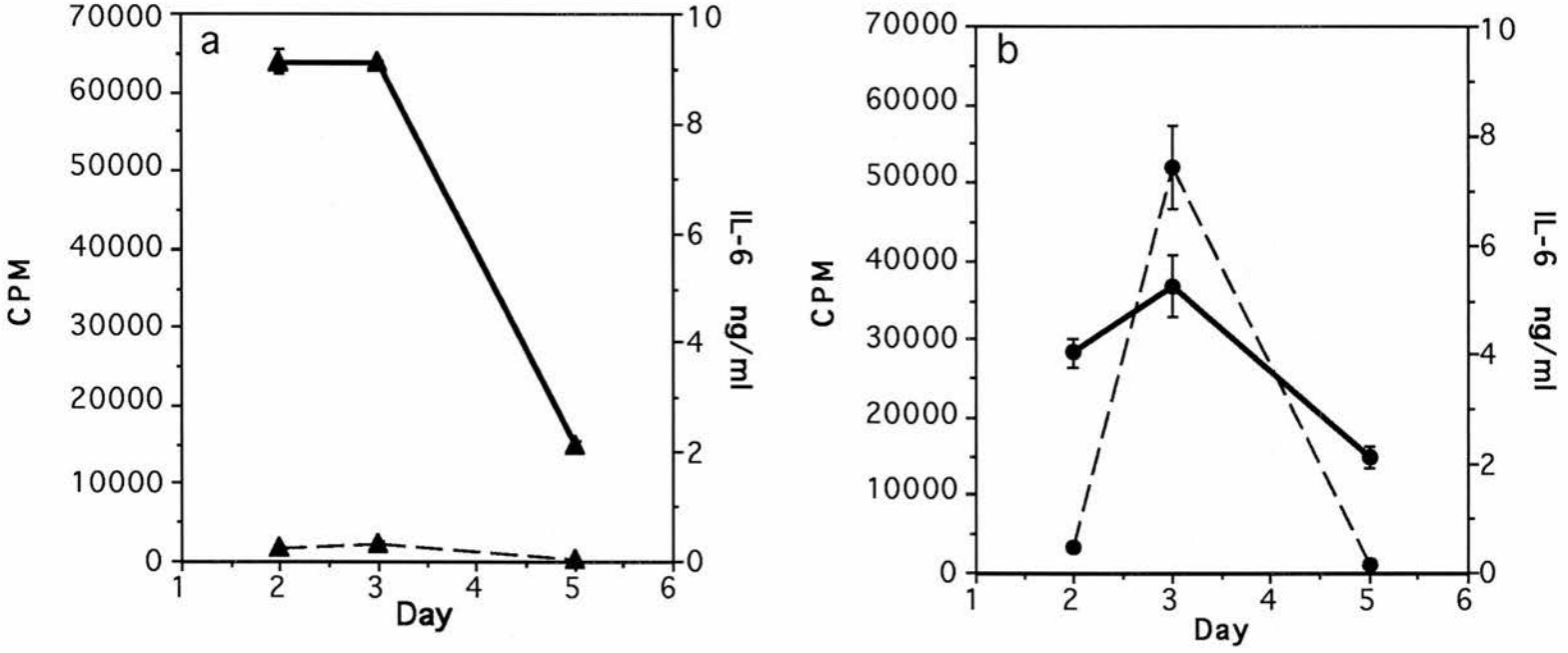

Figure 3.2.3 Kinetics of IL-6 production and proliferative activity of C3H/HeN (Figure a) and BALB/c (Figure b) LNC *in vitro*. LNC were taken from oxazolone treated mice and cultured for 24 hours. Proliferative responses in CPM±SD (solid lines) and IL-6 production in ng/ml±SD (dashed lines) are shown. Limit of detection for IL-6 = 0.15 ng/ml.

	C3H/HeN		BALB/c	
	Proliferation (CPM)	**IL-6 ng/ml**	**Proliferation (CPM)**	**IL-6 ng/ml**
	66730	0.16	36727	8.00
	22838	0.26	30779	8.10
	63816	0.35	72199	7.10
	22265	0.15	20365	10.5
mean±SEM	43912±12347	0.23±0.05	40018±11242	8.42±0.72

Table 3.2.1 Strain differences in LNC proliferative activity and IL-6 production *in vitro* following oxazolone sensitisation. Three days after ear painting with oxazolone auricular lymph nodes were removed, single cell suspensions of LNC prepared and cultured for 24 hours. The proliferative activity was analysed by ^{3}H-thymidine incorporation and supernatants were examined for IL-6 activity by ELISA. The mean CPM (n=5) and mean IL-6 (n=3) levels from four separate experiments are shown for each strain. In the final row these figures have been used to provide the mean proliferative response and IL-6 concentration ± SEM for the four separate experiments for both C3H/HeN and BALB/c mice.

C57BL/6 mice were examined, which are susceptible to UVB-induced immunosuppression in both local and systemic models (Streilein and Bergstresser, 1988; Noonan and Hoffman, 1994). It can be seen (Figure 3.2.4) that oxazolone-induced proliferative responses which peaked on day 2 at 27000 CPM and then dropped to 24000 CPM on day 3 and to 8000 CPM on day 5 following oxazolone treatment. The vehicle-induced (AOO) proliferative response on day 3 was 8700 CPM (data not shown), in other strains vehicle-induced proliferation varied between 2-5000 CPM. It can be seen that C57BL/6 mice produced low levels (<0.4 ng/ml) of IL-6 following oxazolone treatment.

DBA/2 strain mice were tested next, these mice are susceptible to UVB in the systemic model (Noonan and Hoffman, 1994) and resistant in the local model (Streilein and Bergstresser, 1988) of UVB-induced immunosuppression. Again oxazolone-induced good LNC proliferative responses which peaked at around 48000 CPM on day 3 (Figure 3.2.5). The proliferative response of vehicle treated LNC was 17000±243 CPM at the same timepoint, which is very high compared to other vehicle controls (data not shown). However, DBA/2 mice failed to produce appreciable levels of IL-6 following oxazolone-sensitisation (<0.25 ng/ml) or vehicle treatment (<0.15 ng/ml).

Mice of the AKR strain were also tested, which are UVB resistant in the systemic model (Noonan and Hoffman, 1994), and were not examined in the local model (Streilein and Bergstresser, 1988) of UVB-induced immunosuppression. In Figure 3.2.6 oxazolone treatment resulted in LNC proliferative responses which peaked on day 3 following sensitisation (30000 CPM), with LNC from vehicle treated mice proliferating less (7000 CPM) at the same timepoint. However, LNC from oxazolone-sensitised AKR mice produced low levels (<0.45 ng/ml) of IL-6. Table 3.2.2 summarises the responsiveness of the strains to UVB in previous studies and their ability to produce IL-6 following sensitisation.

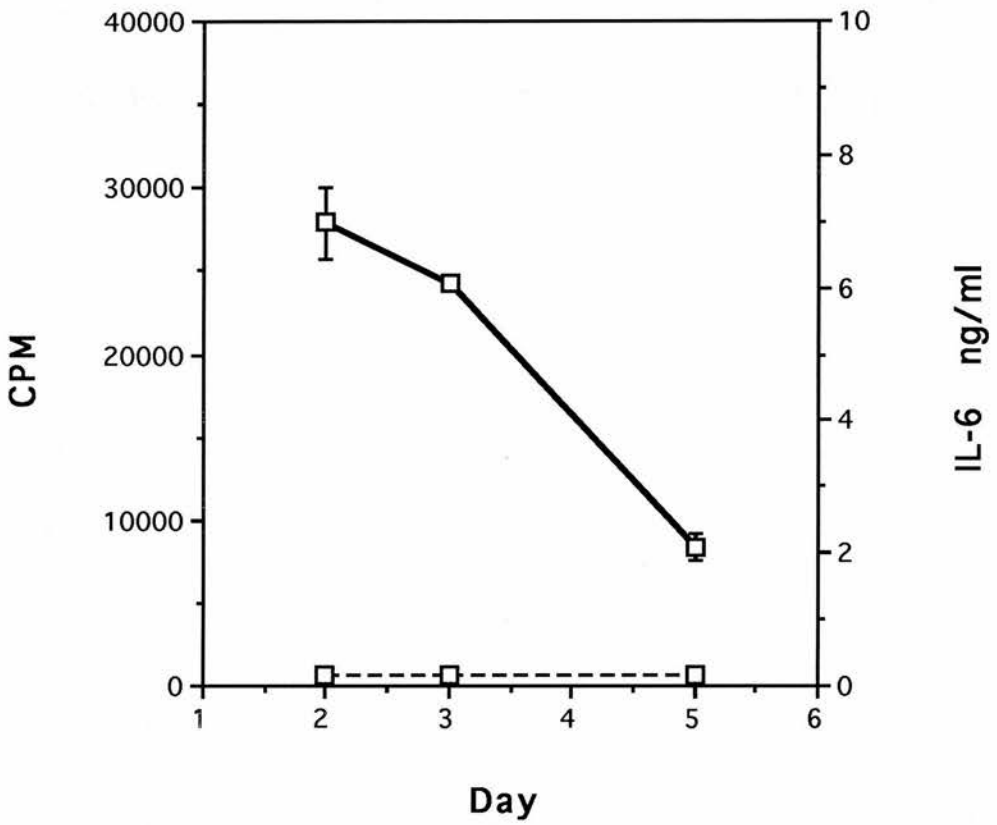

Figure 3.2.4 Kinetics of IL-6 production and proliferative activity of C57BL/6 LNC *in vitro*. LNC were taken from oxazolone treated mice at 2, 3 and 5 days after sensitisation and cultured for 24 hours. Proliferative responses in CPM±SD (solid lines) and IL-6 production in ng/ml±SD (dashed lines) are shown. Limit of detection for IL-6 = 0.15 ng/ml.

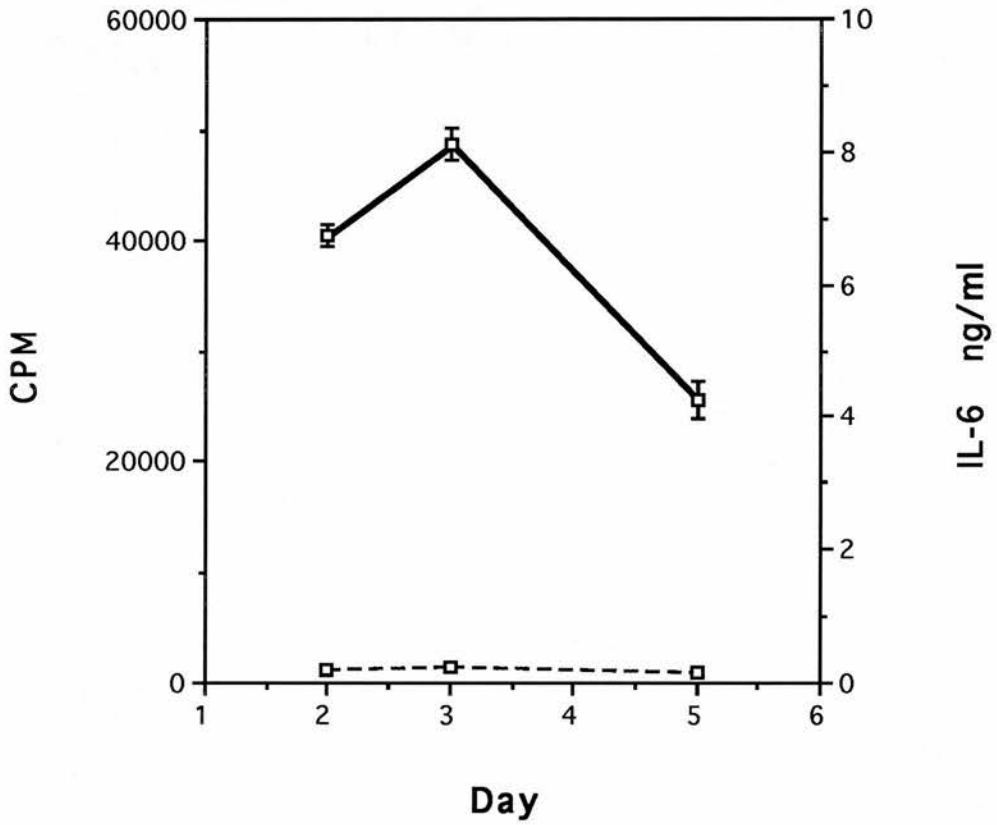

Figure 3.2.5 Kinetics of IL-6 production and proliferative activity of DBA/2 LNC *in vitro*. LNC were taken from oxazolone treated mice at 2, 3 and 5 days after sensitisation and cultured for 24 hours. Proliferative responses in CPM±SD (solid lines) and IL-6 production in ng/ml±SD (dashed lines) are shown. Limit of detection for IL-6 = 0.15 ng/ml.

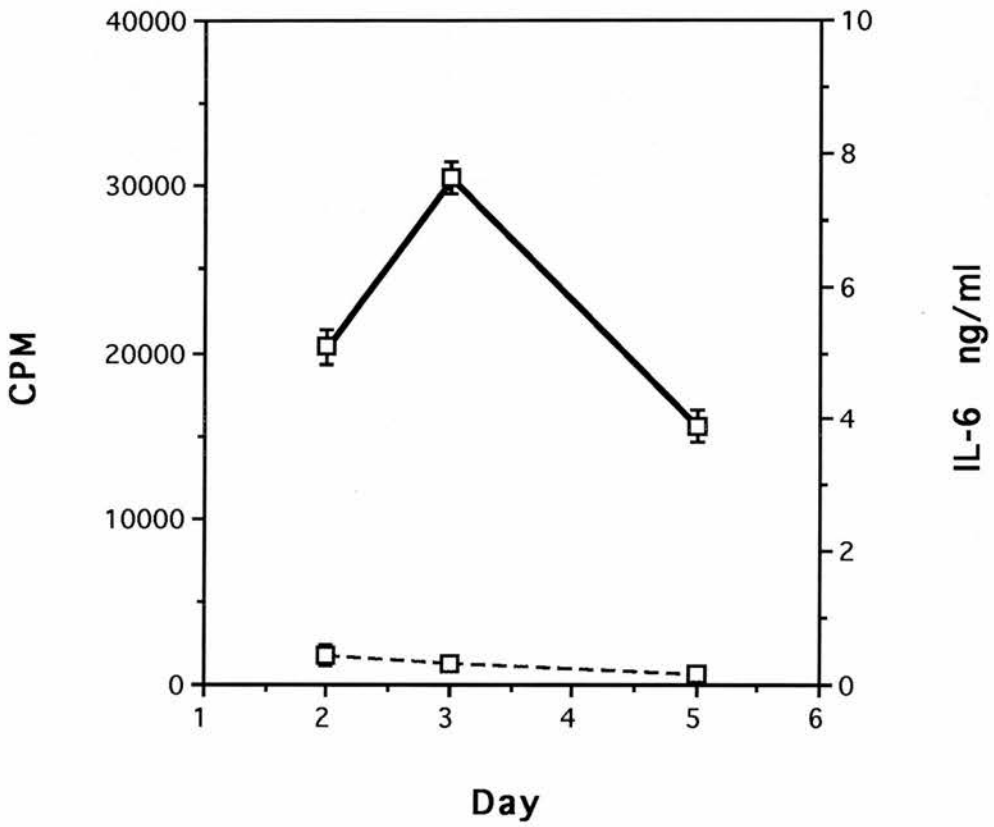

Figure 3.2.6 Kinetics of IL-6 production and proliferative activity of AKR LNC *in vitro*. LNC were taken from oxazolone treated mice at 2, 3 and 5 days after sensitisation and cultured for 24 hours. Proliferative responses in CPM±SD (solid lines) and IL-6 production in ng/ml±SD (dashed lines) are shown. Limit of detection for IL-6 = 0.15 ng/ml.

Strain	local UV suppression	systemic UV suppression	lymphoproliferation (day 3)	IL-6 ng/ml (day 3)
C3H/HeN	+	+	43912±24694	0.23±0.09
C57BL/6	+	+	24253±379	<0.15
DBA/2	—	+	48782±1444	0.23±0.04
BALB/c	—	—	40018±22485	8.42±1.45
AKR	no data	—	30544±1019	0.32±0.08

Table 3.2.2 A summary of reported strain differences in local and systemic UVB-induced immunosuppression, and *in vitro* proliferative responses and IL-6 production by LNC following oxazolone sensitisation.

3.2.4 *Interferon-γ production by LNC*

To determine whether the strain differences in cytokine production were selective for IL-6, the production of IFN-γ by sensitised LNC was examined. In both strains IFN-γ peaked on day 2-3 following oxazolone sensitisation and fell to below detectable levels by day 5 (Figure 3.2.7). Table 3.2.3 shows the oxazolone-induced proliferative responses and IFN-γ levels produced by C3H/HeN and BALB/c LNC in three separate experiments. There was no significant difference in IFN-γ levels in the LNC supernatants of both strains, with C3H/HeN mice producing 0.65±0.13 and BALB/c mice producing 0.95±0.42 ng/ml of IFN–γ. Therefore unlike IL-6, IFN-γ production by LNC was comparable for both C3H/HeN and BALB/c mice.

3.2.5 *DC numbers in naive and sensitised lymph nodes*

Since IL-6 produced by allergen-activated LNC populations is derived predominantly from DC (Hope *et al.* 1995), it was decided to examine DC numbers in the draining lymph nodes of C3H/HeN and BALB/c mice following sensitisation with oxazolone. In both strains, treatment with oxazolone-induced a comparable increase in DC numbers in the DLN 18 hrs later. In C3H/HeN mice DC numbers increased from a mean of 2759 to 23479 per node, whereas in BALB/c mice identical treatment caused an increase in mean DC numbers from 2127 to 22920 per node.

3.2.6 *Intracellular IL-6 in oxazolone-sensitised LNC*

Sonication of LNC taken from both strains 3 days after sensitisation with oxazolone, resulted in detectable levels of IL-6 in the supernatant (Figure 3.2.8). IL-6 levels were significantly higher ($p \leq 0.01$) in LNC from sensitised C3H/HeN and BALB/c mice, compared with LNC from vehicle treated mice. In addition sonication of LNC from sensitised C3H/HeN mice resulted in significantly ($p \leq 0.01$) higher levels of IL-6 being released into supernatant than released from BALB/c LNC after identical treatment.

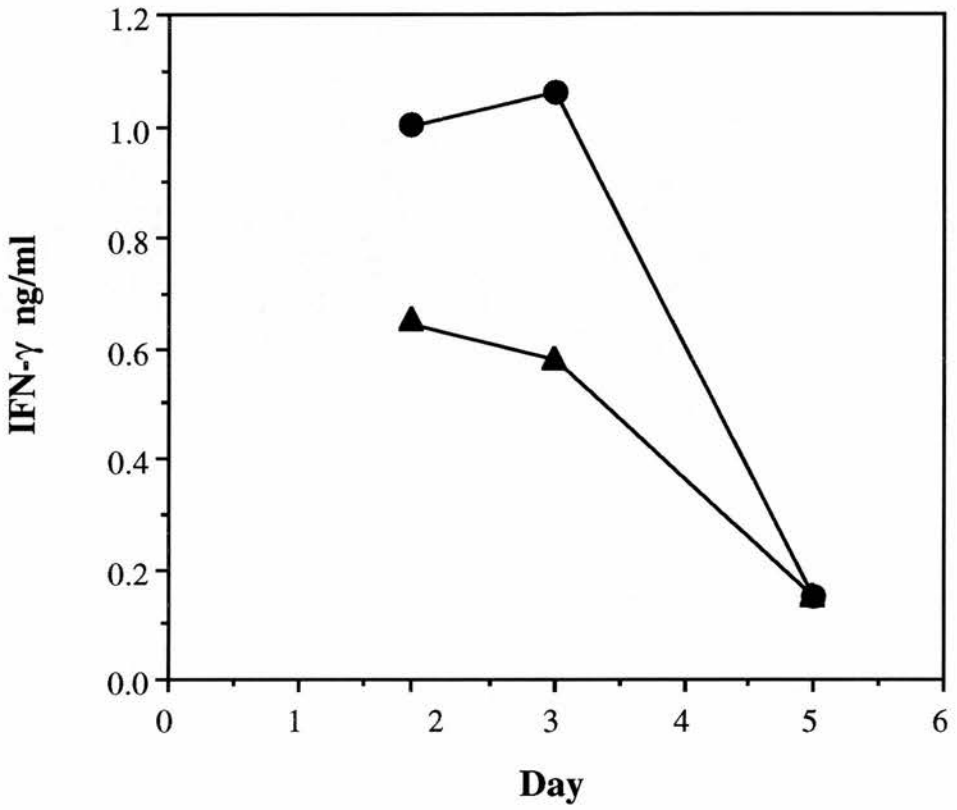

Figure 3.2.7 Kinetics of oxazolone-induced IFN-γ production by C3H/HeN (▲) and BALB/c (●) LNC *in vitro*. LNC were taken from oxazolone treated mice and cultured for 24 hours. IFN-γ levels in ng/ml are shown. Limit of detection for IFN-γ = 0.15 ng/ml.

	C3H/HeN		BALB/c	
	Proliferation (CPM)	**IFN-γ ng/ml**	**Proliferation (CPM)**	**γ ng/ml**
	66730	0.80	30779	1.06
	63816	0.58	34613	0.49
	15338	0.57	72199	1.31
mean±SEM	48628±16666	0.65±0.08	45864±13214	0.95±0.24

Table 3.2.3 Strain differences in LNC proliferative activity and IFN-γ production *in vitro* following oxazolone sensitization. Three days after ear painting with oxazolone, auricular lymph nodes were removed, single cell suspensions of LNC were prepared and cultured for 24 hours. The proliferative activity was analysed by ^{3}H-thymidine incorporation and supernatants were examined for IFN-γ activity by ELISA. The mean CPM (n=5) and mean IFN-γ (n=3) levels from three separate experiments are shown for each strain. In the final row these figures have been used to provide the mean proliferative response and IFN-γ concentration ± SEM for the three separate experiments for both C3H/HeN and BALB/c mice.

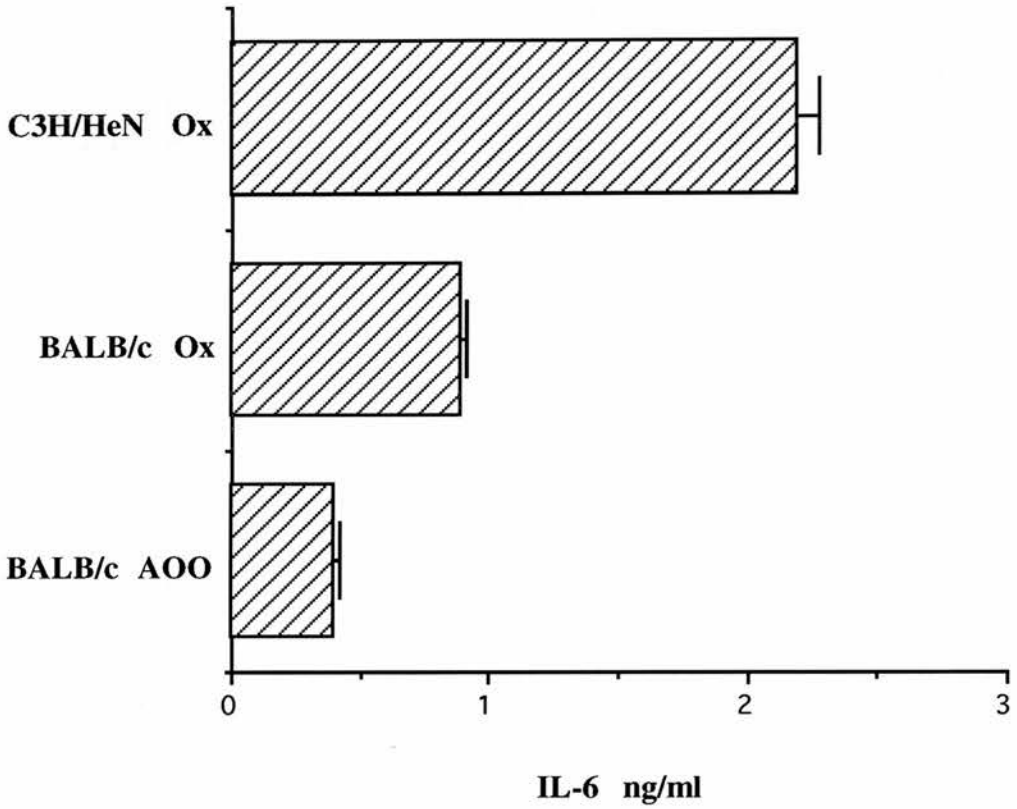

Figure 3.2.8 Intracellular IL-6 content of LNC from C3H/HeN and BALB/c mice. The results show IL-6 levels (ng/ml±SD) in supernatants following sonication of LNC. Draining LNC were removed from mice 3d after skin painting with oxazolone (Ox) or vehicle (AOO). IL-6 was below detectable levels in sonicates of C3H/HeN LNC from AOO treated mice.

In order to investigate further the strain differences in IL-6 secretion, C3H/HeN and BALB/c mice were sensitised with oxazolone and 3 days later LNC were prepared and cultured for 24 hrs. Again, there was a marked difference in the secretion of IL-6 by the two strains, with IL-6 being below the detectable limits in the supernatants from C3H/HeN LNC and BALB/c producing around 4.3 ng/ml of IL-6. Sonication of C3H/HeN and BALB/c LNC after the 24 hr culture period released 0.72 and 2.83 ng/ml of IL-6 respectively into solution. Because the lack of IL-6 secretion by C3H/HeN LNC in culture is not associated with the accumulation of IL-6 within these cells, it seems likely that the strain difference in IL-6 secretion reflects a lack of IL-6 protein production by C3H/HeN LNC.

3.2.7 IL-6 production and intracellular levels of IL-6 following UVB

Exposure to a single dose of 1440 J/m^2 UVB results in the accumulation of DC in the DLN peaking around 48 hrs after exposure (Moodycliffe *et al.*1994). In both strains, exposure to a single dose of 1440 J/m^2 of UVB caused an increase in DC numbers in DLN. In C3H/HeN mice DC numbers rose from 2083 to 7524 per LN, and from 2530 to 6223 per LN in BALB/c mice.

Although UVB causes the accumulation of DC and the induction of LNC proliferation in the DLN, there was no induction of IL-6 activity in either strain. In C3H/HeN mice IL-6 levels were below the detectable limits at all timepoints. In BALB/c mice IL-6 levels were just above the detectable limits on day 1 and 2 (0.25±0.07 and 0.29±0.09 ng/ml respectively) but fell to below the detection limits by day 5.

In separate experiments LNC suspensions prepared from the auricular lymph nodes of C3H/HeN and BALB/c mice 3 days after a single dose of UVB (1440 J/m^2) were sonicated. UVB treatment of C3H/HeN mice raised the intracellular IL-6 levels

from below the detectable limits to 0.17±0.03 ng/ml . In BALB/c IL-6 levels were increased from 0.18±0.01 to 0.32±0.01 following UVB treatment.

3.2.8 IL-6 production in the skin of C3H/HeN and BALB/c mice

IL-6 is produced constitutively by epidermal LC (Schreiber *et al.*1992) and keratinocytes can be induced to synthesise IL-6 (Bos and Kapsenberg, 1993). Figure 3.2.9 shows that similar amounts of IL-6 were produced constitutively in the skin by both C3H/HeN mice (3.84±0.21 mean ng/g tissue±SEM, n=4, range=0.95 ng/g) and BALB/c mice (3.37± 0.29 mean ng/g tissue±SEM, n=4, range=1.29 ng/g). Painting the skin of C3H/HeN or BALB/c mice with vehicle did not cause a substantial increase in IL-6 production over constitutive levels. In C3H/HeN mice, sensitisation with 1% oxazolone induced significant increases (when compared with vehicle controls) in IL-6 levels ($p \leq 0.025$) at all timepoints except 0 hr. In BALB/c mice, oxazolone induced a significant increase ($p \leq 0.025$) in IL-6 levels compared with the vehicle control at all timepoints. The kinetics but not the magnitude of IL-6 production were similar in both strains, with IL-6 levels in both strains peaking 4-8 hrs post-sensitisation and decreasing by 24 hrs. C3H/HeN mice produced higher levels of IL-6 following sensitisation than BALB/c mice. At the peak 4 hr timepoint, skin from C3H/HeN mice contained almost twice as much IL-6 as skin from BALB/c mice (36 ng/g and 22 ng/g tissue respectively). This reflected an increase in IL-6 levels of 40 and 18 ng/g tissue in C3H/HeN and BALB/c mice, respectively following oxazolone sensitisation.

This experiment was repeated (Figure 3.2.10). The kinetics of cytokine induction in this experiment were similar to those shown previously (Figure 3.2.9), with IL-6 activity in both strains peaking at 4-8 hrs after sensitisation. Vehicle treatment failed to cause substantial increases in IL-6 activity, except in BALB/c mice where there were significant ($p \leq 0.025$) increases in IL-6 activity in the skin 0 and 1 hrs following vehicle treatment when compared with levels in untreated mice. Unlike the previous experiment,

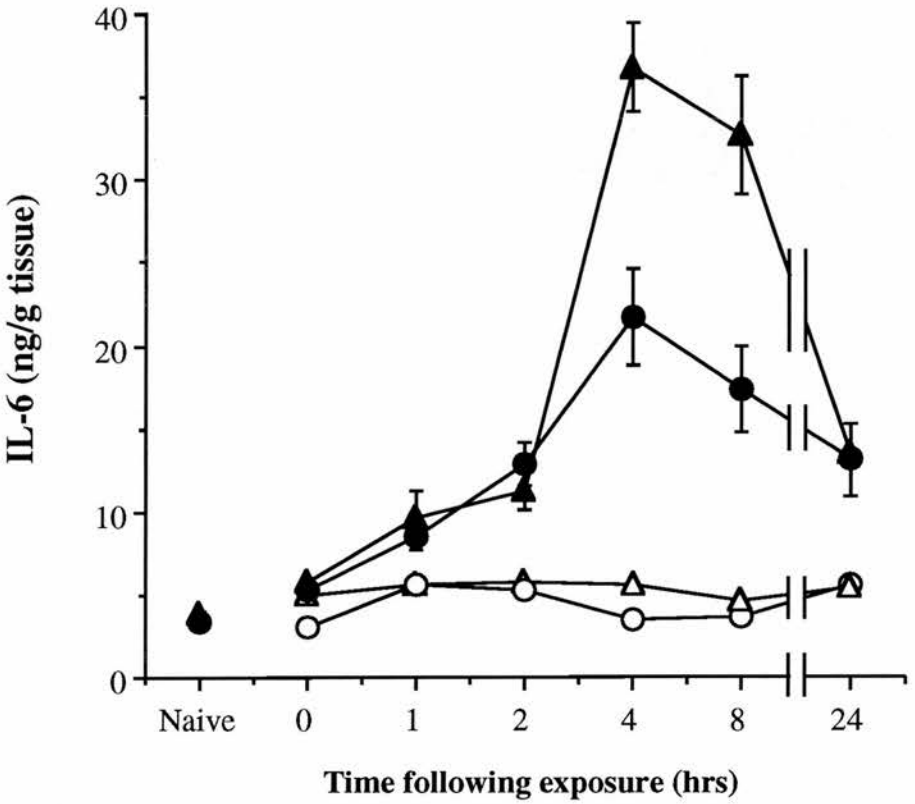

Figure 3.2.9 Kinetics of IL-6 production in the skin of C3H/HeN (triangles) and BALB/c mice (circles) after painting with oxazolone (closed) and AOO (open), naive mice were unpainted. IL-6 levels are shown as ng per gram tissue±SD. Limit of detection for IL-6 = 0.15 ng/ml.

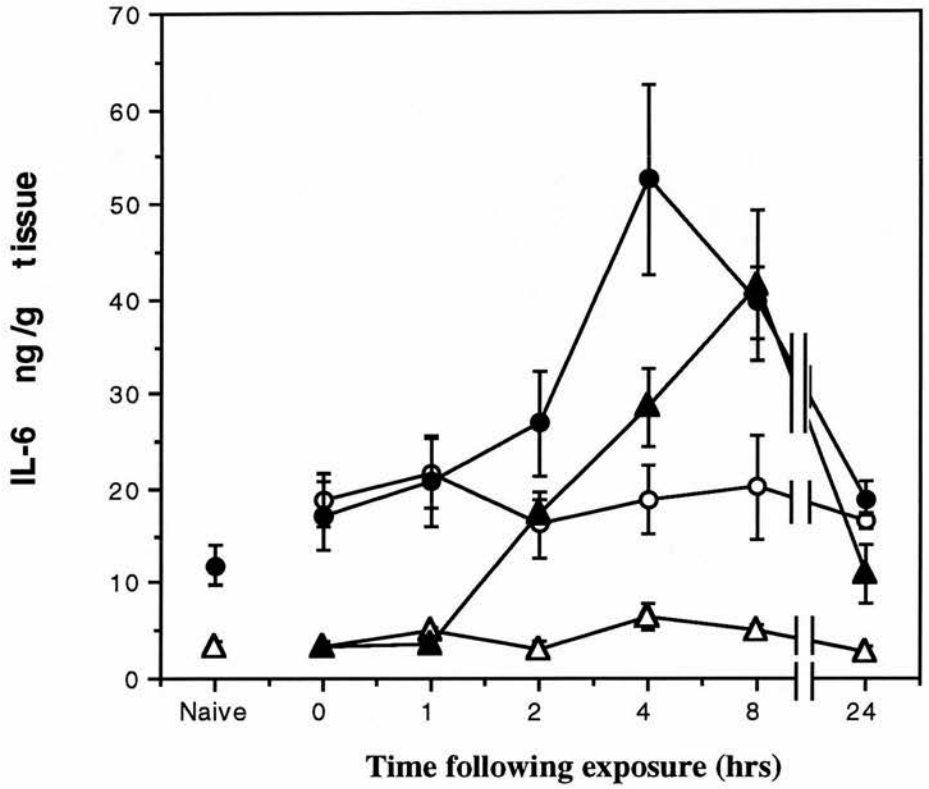

Figure 3.2.10 Kinetics of IL-6 production in the skin of C3H/HeN (triangles) and BALB/c mice (circles) after painting with oxazolone (closed) and AOO (open), naive mice were unpainted. IL-6 levels are shown as ng per gram tissue±SD. Limit of detection for IL-6 = 0.15 ng/ml.

the constitutive IL-6 levels were significantly (p=0.025) higher in the skin of BALB/c mice compared with C3H/HeN mice. In addition, the magnitude of IL-6 production was generally higher in BALB/c mice than C3H/HeN mice, significantly ($p \leq 0.025$) at 1, 4, and 24 hrs. The increase in IL-6 levels following oxazolone sensitisation was 35 and 30 ng/g tissue in C3H/HeN and BALB/c mice, respectively at the 4 hr timepoint. These levels were similar to those seen in the previous experiment (Figure 3.2.9).

3.2.9 The effect of UVB on IL-6 production in the skin

Because the cutaneous microenvironment is an important component in the skin immune system, the effect of UVB on IL-6 activity in the skin was examined. In the initial experiment C3H/HeN and BALB/c mice were either unirradiated or exposed to UVB (1440 J/m^2, -48 and -24 hrs) prior to sensitisation (Figure 3.2.11). The effect of UVB on constitutive, vehicle and oxazolone-induced IL-6 production was measured 4 hrs after the appropriate sensitisation treatment.

The magnitude of the 4 hr oxazolone responses (Figure 3.2.11) was comparable to those seen in Figure 3.2.9 with C3H/HeN mice producing around 30 ng/g tissue weight (around 35 ng/g previously) and BALB/c mice producing around 14 ng/g tissue weight (compared with 21ng/g IL-6 in Figure 3.2.9). From this experiment it appears that UVB is having a differential effect on IL-6 in the skin of C3H/HeN and BALB/c mice. IL-6 production in naive, vehicle treated and oxazolone-treated and UVB irradiated mice was significantly increased (p=0.025) in C3H/HeN but not BALB/c mice.

However repeating the experiment failed to confirm the findings (Figure 3.2.12). The magnitude of the response was much higher than in previous experiments with oxazolone inducing 107 and 79ng of IL-6 per gram tissue in C3H/HeN and BALB/c mice respectively. There was no statistically significant inter-strain difference in the effect of UVB on constitutive, AOO and oxazolone-induced IL-6. UVB caused a significant

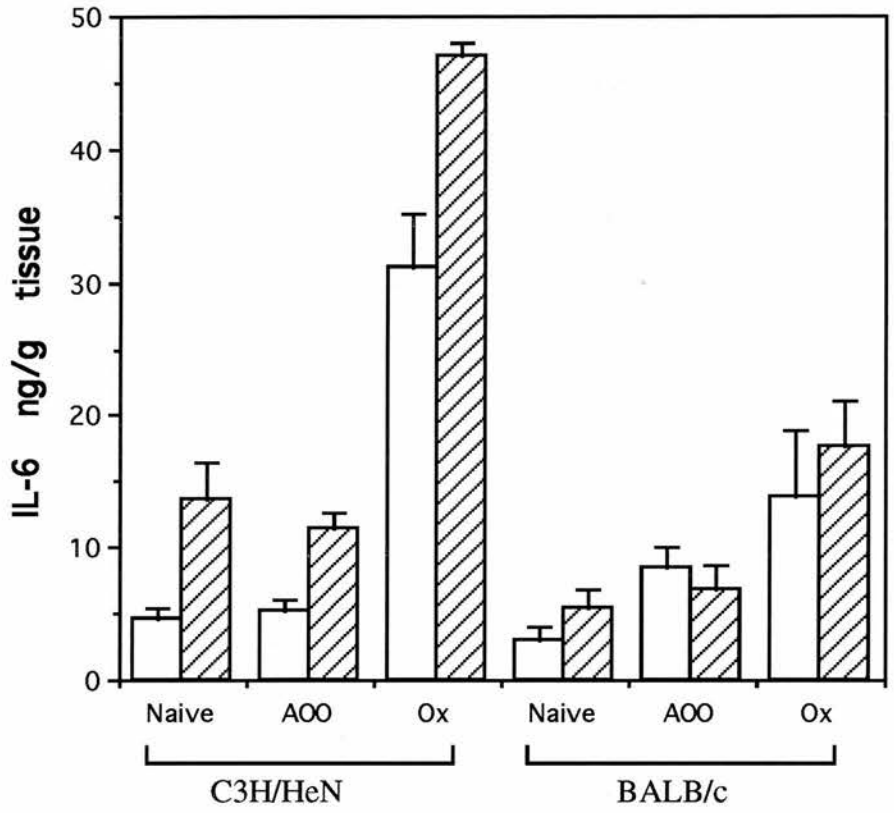

Figure 3.2.11 The effect of UVB exposure on constitutive and oxazolone-induced IL-6 production in the skin of C3H/HeN and BALB/c mice. The shaved backs of mice were not irradiated (□) or were exposed to UVB (1440J/m^2, -48 and -24 hrs) (▨) prior to sensitisation. Mice were then left untreated (naive), were sensitised with vehicle (AOO) or with oxazolone (Ox). Skin samples were taken at 4 hrs following sensitisation, weighed, processed and analyzed for IL-6. The results are given as nanograms of IL-6 per gram of tissue weight.

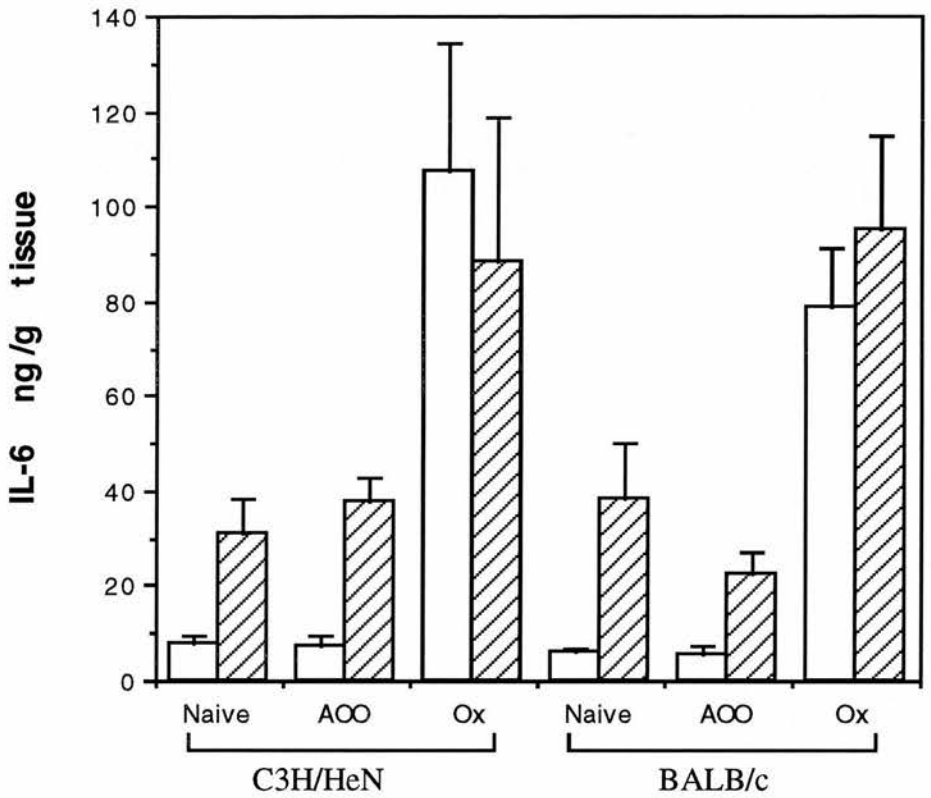

Figure 3.2.12 The effect of UVB exposure on constitutive and oxazolone-induced IL-6 production in the skin of C3H/HeN and BALB/c mice. The shaved backs of mice were not irradiated (□) or were exposed to UVB (1440J/m^2, -48 and -24 hrs) (▨) prior to sensitisation. Mice were then left untreated (naive), were sensitised with vehicle (AOO) or with oxazolone (Ox). Skin samples were taken at 4 hrs following sensitisation, weighed, processed and analyzed for IL-6. The results are given as nanograms of IL-6 per gram of tissue weight.

increase (p=0.025) in constitutive and AOO-induced IL-6 activity , but failed to significantly increase oxazolone-induced cutaneous IL-6 in either strain.

The wide differences in the magnitude of IL-6 levels in the skin may reflect differences in the protocol used. The protocol used for the first kinetics experiment and the first UVB experiment (Figures 3.2.9 and 3.2.11) differed from the protocol used to repeat these respective experiments (Figures 3.2.10 and 3.2.12). In the first experiments the skin samples from 4 mice (0.5-1g) were chopped up, pooled and stored at -70°C before they were homogenised and sonicated. Following discussion (personal communication Dr. R Dearman) it was decided that lower weights of skin between 0.05 and 0.1g would be more suitable for analysis. Therefore before the homogenisation, skin from each sample was blotted dry and known weights of sample redistributed to four eppendorfs. In the later experiments (Figures 3.2.10 and 3.2.12), the samples from each of the four mice were processed separately and at no point in the proceedings were samples pooled. The effect of the protocol change on IL-6 levels will be discussed later, but briefly the increased processing time, extra freeze/thaw cycles and the innaccuracies incurred by re-weighing samples may possibly be reflected in the lower levels of IL-6 detected in Figures 3.2.9 and 3.2.11 compared with Figures 3.2.10 and 3.2.12.

3.2.10 Summary

The induction phase of CH is associated with proliferation (Scholes *et al.* 1992) and IL-6 production by LNC draining the sensitised site (Hope *et al.* 1994). Accumulating DC provide the main source of IL-6 within the sensitised lymph node (Hope *et al.* 1995; Cumberbatch *et al.* 1996). Since IL-6 is important in the early stages of antigen presentation (Van Snick, 1990), it is possible that its production by DC is important in the generation of effector T lymphocyte populations. Consistent with previous studies (Hope *et al.* 1994), it was shown that LNC from UVB-resistant BALB/c mice proliferated and produced high levels of IL-6 in response to the sensitiser, oxazolone. In contrast, LNC from UVB-susceptible C3H/HeN mice produced low-levels

of IL-6 following sensitization but showed similar proliferative activity to BALB/c LNC. The lack of IL-6 activity was not attributable to a congenital variation between these strains with respect to IL-6 production as comparable levels of constitutive and inducible expression of this cytokine were measured in BALB/c and C3H/HeN mice, following respectively, topical exposure to vehicle alone or oxazolone. Sonicating LNC from sensitised BALB/c and C3H/HeN mice, immediately after removal from the mice, caused the release of similar levels of IL-6. The total amount of IL-6 produced by LNC during 24 hrs, was measured by sonicating LNC following culture and by analysing the levels of IL-6 in culture supernatants. The failure of C3H/HeN LNC to secrete IL-6 into culture was not reflected by the accumulation of the cytokine within cells. Therefore it seems likely that the low levels of IL-6 produced by C3H/HeN LNC reflect a lack of IL-6 production by LNC, rather than a failure in IL-6 secretion. To clarify the role of IL-6 production in UVB-induced immunosuppression, further UVB-resistant (AKR) and UVB-susceptible (C57BL/6 and DBA/2) strains were examined, all of which showed low levels of IL-6 production following sensitisation with oxazolone. Therefore no correlation was shown between the levels of IL-6 production following sensitisation, and the susceptibility or resistance of mice to the immunosuppressive effects of UVB.

Chapter 4. Discussion

4.0 Chronic UVB

4.0.1 The effect of UVB exposure on LC density in the epidermis

Exposure to single doses of broadband UVB or TL01 has been reported to decrease the density of LC in the epidermis (El-Ghorr *et al.*1994). Similarly chronic UV exposure, by the broad- and narrowband sources, reduced the density of ATPase$^+$ LC throughout the period of irradiation (Figure 3.0.2). The reduction was dose dependent for the broadband source, with the higher dose (1000 J/m^2) causing the greatest loss in LC numbers at all timepoints examined. For mice exposed to the broadband source, LC dropped to a minimum during the first 2-3 weeks of chronic exposure. After this LC numbers began to rise, suggesting that LC may have started to repopulate the epidermis, but they had not reached pre-irradiation levels by 6 weeks. Other studies have reported that the LC density returned to normal levels after about 15 weeks of chronic UVB treatment (Alcalay *et al.*1989). The TL01 lamp was less effective than the broadband lamp at reducing the density of LC at early timepoints, but by 6 weeks the numbers had decreased to a similar extent as with the higher-dose (1000 J/m^2) broadband source.

4.0.2 The effect of UVB on sunburn cell (SBC) induction

SBC are thought to represent apoptotic keratinocytes and are induced in the mammalian epidermis in a UV-dose dependent manner (Young, 1986; Woodcock and Magnus, 1976). SBC were found during chronic irradiation with both broad- and narrow band sources (Figure 3.0.4). They were induced in a dose-dependent manner by broadband UVB. Fewer SBC were formed after TL01 exposure compared with broadband UVB. The induction of SBC is wavelength dependent, peaking between 260-280nm and droping dramatically from 290 to 310nm (Woodcock and Magnus, 1976).

Because SBC induction plateaus between 260-280nm it is not possible on this evidence to separate DNA or protein as the main chromophore involved. TL01 has a restricted emission spectrum and is likely to be less effective than broadband UVB in the induction of SBC, though in this study it was only slightly less effective than the lower dose (500 J/m^2) of broadband UVB (Figure 3.0.4).

Following acute exposure to around one MED (1500 J/m^2 UVB), 24hrs previously, 5.4 SBC/cm of epidermis were found, while in BALB/c mice, exposure to 2000 J/m^2 of broadband UVB resulted in around 70 SBC/cm of epidermis 24 hrs later (Schwartz *et al.* 1995). Therefore, the numbers of SBC found in this study following chronic irradiation seem very low (Figure 3.0.4). However, in the protocol, the ears were removed from the mice three days after the last irradiation. In other studies peak numbers of SBC were found 8h after UVC and 24 hrs after UVB exposure (Young, 1986). The peak values must represent the minimum time to SBC formation and clearance, though the mechanism for SBC removal is unclear. The most obvious route would be removal by desquamation. However the minimum transit time of cells from the basal layer to the surface is 6 days (Potten, 1975). Although in this study SBC were observed supra-basally, the rapid removal of SBC suggests a more dynamic clearance mechanism, and, there is evidence for keratinocyte-mediated phagocytosis of SBC (Young, 1986). Whatever the mechanism of SBC removal, it seems likely that the 3 day gap between the last UVB exposure and the preparation of skin sections allowed clearance of some SBC, and resulted in the low numbers of SBC identified after chronic UVB.

4.0.3 Chronic UVB, skin ageing/damage and epidermal thickness

Chronic low dose UVB exposure results in photoageing which is marked by histological and biochemical changes in the skin (Kligman, 1991). The appearance of photoaged skin reflects UVB-induced damage to collagen and glycoaminoglycans (GAG) in the dermis (Kligman, 1991). In hairless Skh mice, most of the features of photoageing

(wrinkling, skin fold and epidermal thickening, collagen damage, GAG increases, increased dermal cellularity) are induced efficiently by wavelengths of between 290-300 J/m^2 in the UVB range (Bissett *et al.*1989). Only one measure of photoageing, skin sagging, is dependent on exposure to UVA with a peak induction at 340nm.

The effect of multiple exposures of C3H/HeN mice to broad- and narrowband UVB was investigated. While each dose of irradiation was suberythemal, it was noted that, after 2 weeks' exposure of mice to 1000 J/m^2 of the broadband source, the skin showed some reddening, before pigmentation developed. Exposure to 500 J/m^2 of broadband UVB did not produce erythema, although tanning was evident after 3 weeks. The narrowband TL01 lamp did not produce reddening or tanning.

The TL01 lamp was developed for use in phototherapy treatment, to minimize the damaging effects of UV light (Van Weelden *et al.*1988). Because the TL01 lamp emits a very narrow peak around 311-312 nm, exposure to narrowband UV causes less skin damage than broadband sources. Previous studies have shown broadband UVB to be more efficient than UV from the TL01 source in the induction of epidermal thickening (Sternborg and van der Leun, 1986). This was confirmed in our study with chronic exposure to 1000 J/m^2 of broadband UVB causing a doubling of epidermal thickness, while TL01 exposure failed to increase epidermal thickness (Figure 3.0.5).

4.0.4 UVB exposure and cis*-UCA concentration*

UCA in the stratum corneum is isomerized by UVB from the naturally occurring *trans* form to *cis*-UCA. A number of studies have suggested *cis*-UCA to be a mediator of UVB-induced immunosuppression. Some of these studies were discussed in the introduction (section 1.8.2.2).

UVB causes a dose dependent conversion of UCA to the *cis*-isomer both *in vitro* and *in vivo* until the photostationary state is reached, when approximately 50% of the total UCA is in the *cis* form (Morrison *et al.*1980). Interestingly in this study, *cis*-UCA seems not to have reached a photostationary state. The concentration of *cis*-UCA peaked

at 1-2 weeks of chronic broadband UVB (1000 J/m^2) and TL01 exposure (Figure 3.0.6) at which point *cis*-UCA represented around 35-38% of total UCA . However, even though UVB exposure was continued up to 6 weeks the concentration of *cis*-UCA remained constant in mice exposed to broadband UVB, and dropped in those exposed to the TL01 source (Figure 3.0.6). The drop in *cis*-UCA levels in TL01 exposed mice occurred in the absence of epidermal thickening (Figure 3.0.5) or tanning. Following the formation of *cis*-UCA in the epidermis, it has been shown to persist in the epidermis for 7 days, returning to background levels by 14 days (Norval *et al.*1988). It is eliminated by desquamation and in sweat, but the increased solubility of the *cis*-isomer compared with the *trans*-UCA means that *cis*-UCA is found transiently in the serum of mice following UVB (Moodycliffe *et al.*1993).

Previous studies have shown an increase in total UCA in the skin of humans and guinea pigs respectively following insolation and irradiation with a hot quartz mercury arc lamp (Hais *et al.* 1970, Baden and Pathak, 1967). These increases occurred several days after irradiation and were thought to correlate with the increased epidermal thickness of irradiated skin. Interestingly although broadband UVB caused an increase in epidermal thickness (Figure 3.0.5) the total UCA concentration remained unchanged throughout the study (Figure 3.0.6). This agrees with results from a human study which showed comparable concentrations of total UCA in epidermal sites which are commonly exposed to the sun, such as the forehead, compared with sites that receive little sun exposure, such as the upper thigh (Kavanagh *et al.* 1995).

4.0.5 Chronic UVB and CH responses.

CH responses to oxazolone were suppressed to a similar extent in mice exposed to 500 and 1000 J/m^2 of broadband UVB for six weeks (Figure 3.0.7). In contrast, TL01 exposure for 3 or 6 weeks did not cause a suppression of CH responses (Figure 3.0.7). The failure of TL01 exposure to suppress CH responses has also been observed following acute exposure (El-Ghorr *et al.*1994). In this study CH responses were elicited on the ears of mice, which were not protected during UVB exposure. This is more likely to reflect environmental UVB exposure and has previously been shown not to affect CH responses following acute UVB. However, the effect, of protecting the ears during chronic broadband UVB exposure on CH responses were not examined in this study. Therefore it is impossible to distinguish between UVB-induced effects on the induction versus the elicitation of CH responses.

Both broad- and narrowband sources reduced LC density by around 50% by week 6 of exposure (Figure 3.0.2). Since broadband but not TL01 exposure resulted in suppression of CH responses (Figure 3.0.7), it seems unlikely that the reduction in epidermal LC density alone is responsible for the loss of CH responses following UVB. Similarly the fact that CH responses were not suppressed at 3 (data not shown) or 6 weeks in TL01 exposed mice (Figure 3.0.7), even though the levels of *cis*-UCA after 3 weeks of TL01 exposure were similar to the maximum levels induced by broadband UVB (Figure 3.0.6), suggests that *cis*-UCA is not the main mediator of chronic UVB-induced suppression of CH.

There has been relatively little work studying the effects of chronic UVB exposure on the SIS. However, a chronic low-dose protocol is far more likely to reflect the type of exposure that individuals receive from terrestrial sunlight. One of the interesting results from this study was the adaptation of murine skin to chronic UVB exposure. Chronic UVB failed to deplete completely LC from the skin, and by week 5 and 6 numbers were

beginning to return to normal in mice exposed to broadband UVB. SBC numbers peaked after 1-2 weeks of UV exposure and by 6 weeks were returning to the levels seen in un-irradiated mice. The other marker of photodamage, increased epidermal thickness, reached a plateau 1-3 weeks into broadband UVB exposure. Finally *cis*-UCA, which has been been shown to reach its photostationary state following acute irradiation both *in vitro* and *in vivo*, failed to reach this level during chronic exposure. The most likely explanations for this ability of murine skin to adapt to UVB is the epidermal thickening and pigmantation induced by the broadband UVB source. The TL01 source failed to induce either epidermal thickening or tanning, which may explain why LC numbers were still dropping in the latter stages narrowband exposure. Interestingly however, SBC began to return to normal levels by 5 weeks of TL01 exposure, and the level of *cis*-UCA in the mice remained stable after the first week of narrowband UV exposure. It is possible therefore that chronic narrowband exposure induces an adaptive response from the skin separate from epidermal thickening or pigmentation.

4.1 UVB exposure and DC function and phenotype

4.1.0 In vivo UVB exposure and DC function

UVB exposure causes the suppression of cutaneous immune responses to a variety of agents. Of particular relevance to this study is the ability of UVB to suppress immune responses to contact sensitisers. The importance of DC in UVB-induced suppression of CH responses was apparent from the initial experiments in this area by Toews *et al.* (1980). They demonstrated the failure of CH induction through sites naturally deficient in LC, such as mouse tail skin. This observation was extended in the same study by exposing the shaved skin of mice to UVB on 4 consecutive days, a treatment which resulted in a depletion of LC from the exposed site. Like skin that was naturally deficient in LC, sensitisation through skin exposed to UVB, did not result in the induction of CH responses. This work suggested that it was the density of LC in the skin that determines whether immune responses will be induced to particular antigens. There is no doubt that LC density is important in the induction of immune responses, but the examination of UVB-resistant mice has suggested that other factors are also critical. In both UVB susceptible and resistant strains, exposure to low-doses of UVB causes the depletion of LC from the skin, but only in susceptible strains does this LC depletion result in suppression of cutaneous immunity (Streilein and Bergstresser, 1988). There is growing evidence that dermal cells in UVB resistant but not susceptible mice provide a secondary pathway of CH induction following exposure to UVB. Dermal cell suspensions prepared from C57BL/6 (UVB susceptible) and BALB/c (UVB resistant) mice and haptenated *in vitro* transferred CH responses to syngeneic mice (Kurimoto *et al.*1994). Complement depletion experiments showed that the transfer of CH responses in this model was dependent on dermal MHC class II^+ cells. Exposure of mice to

400 J/m^2 of UVB on 4 consecutive days suppressed the ability of dermal cells from C57BL/6 but not BALB/c mice to transfer CH responses to syngeneic recipients (Kurimoto *et al.* 1994).

4.1.1 Functional changes in LC

Because the loss of LC from the skin fails to explain fully the immunosuppressive effects of UVB, it was of interest to examine the effects of UVB on the antigen presenting activity of DC *in vivo*. Murine DC were enriched from the auricular lymph nodes, which drain the ears, following a variety of treatments. In mice the ears provide a useful surface for skin treatments. First they offer an easily accessible, hairless and semi-discrete site for exposure of mice to UVB and chemical allergens. Secondly, treatments which cause the loss of LC from the ears result in the accumulation of DC in the auricular lymph nodes (Macatonia *et al.* 1987). This being the case there is the possibility of examining changes in APC populations initiated in the skin by UVB exposure, at the level of DC in the DLN. A system was developed therefore in which an immunosuppressive dose of UVB was given to mice prior to sensitisation using either FITC or oxazolone. The APC activity of DC isolated from mice that received UVB from the DLN prior to sensitisation was compared with the APC activity of sensitised DC from non-irradiated mice.

4.1.2 Are DC populations in skin draining lymph nodes derived from LC?

Identifying UVB-induced changes in DC accumulating in the DLN assumes that at least some of these cells derive from cutaneous DC. The techniques that have been used to study this are limited, particularly in mice because of the inability to examine the lymphatic drainage of their skin. The migration of LC out of sensitised skin of mice has been investigated using the fluorescent chemical allergen FITC (Macatonia *et al.* 1987). In addition UVB-induced LC migration has been examined by identifying DC with thymine dimers in the DLN (Sontag *et al.* 1995).

Macatonia *et al.* (1987), reported two populations of $FITC^+$ DC in the DLN after skin painting, one with low levels of FITC and another intensely fluorescent population. DC with high fluorescent intensity first appeared 2-8 hrs after skin painting and induced T cell proliferation. Kripke *et al.* (1990), reported two populations of $FITC^+$ DC in the DLN after skin painting; 75% of these DC expressed the antigen F4/80, a marker of LC but not lymph node DC, which suggested that they are a skin-derived population. The remaining 25% of the DC did not express F4/80 and may represent a resident lymph node population. In agreement with the studies of Macatonia *et al.* (1987), ear painting with FITC resulted in the appearance of FITC-bearing cells in the DLN with the size and granularity of DC (Figure 3.1.3b). In addition in Figure 3.1.3b, two distinct populations of DC can be seen which show low and high fluorescent intensity, which is similar to the report of Macatonia *et al.* (1987). It is possible that free, non cell-associated FITC reaches the lymph node following skin painting. Cells with low-intensity fluorescence may therefore represent a resident lymph node population which have bound FITC *in situ*. Certainly they appear rapidly (30 min) following skin painting and are unable to induce FITC specific responses (Macatonia *et al.*1987). However epicutaneous exposure to FITC caused the accumulation of DC systemically in both local and distant lymph nodes (Hill *et al.* 1990), though only DC in DLN expressed FITC, and stimulated FITC-specific T cell responses.

Contrasting results were obtained in a study using a monoclonal antibody to thymine dimers to identify DC with UVB-induced DNA damage in the DLN of hairless mice (Sontag *et al.*1995). Less than 0.3 cells per 1000 enriched DC showed thymine dimers. Although the study did not report the numbers of DC in lymph nodes following UVB, assuming that UVB causes an increase in DC numbers from around 2500 to 10000 DC per LN (Moodycliffe *et al.* 1992) and that all skin-derived DC show thymine dimers, then the result suggests that only around 3 DC accumulating in the DLN following UVB were skin derived. This seems fairly unlikely and suggests that the assumptions are

flawed. Since the first assumption results from published observations, it seems likely that the second assumption, that all skin-derived DC should exhibit DNA damage, needs rethinking. The study was carried out in hairless HRA/skh mice, and it would be of considerable interest to carry out the same study in DNA repair deficient mice. In normal inbred mouse strains it is impossible to know whether the low numbers of DNA damaged DC in DLN reflect a low level of migration from the skin, or effective DNA repair. However a second possibility exists, that the ability of LC to migrate out of the skin in response to UVB exposure means that the DC accumulating in the DLN will be unlikely to show DNA damage whether they are from normal or DNA repair deficient mice.

Because of the technical limitations involved in using monoclonal antibodies to thymine dimers to identify LC-derived DC, it seems likely that the evidence from the FITC studies more accurately reflects the numbers of LC-derived DC in the DLN. The FITC studies are in line with those in sheep (Dandie *et al.*1992) which have shown large numbers of $CD1a^+$ LC, migrating via the afferent lymph following treatment of skin with chemical allergens and carcinogens. Though still a point of controversy, it seems likely that the majority of DC in the DLN after skin painting with a contact sensitiser is skin derived. Therefore it was considered that they constitute an appropriate APC population with which to examine the immunomodulatory effects of UVB *in vivo*.

4.1.3 UVB-induced suppression of cutaneous immune responses

In order to examine the effect of UVB treatment *in vivo* it was important to expose mice to sufficient UVB to cause suppression of CH responses. Previously it has been shown that exposure of C3H/HeN mice to 2 doses of 1440 J/m^2 of UVB 48 and 24 hrs prior to sensitisation results in around a 50% suppression of CH responses (Moodycliffe *et al.*1994). In the present study, exposure to the same dose of UVB caused significant suppression of CH responses on two separate occasions (Figure 3.1.1 and Figure 3.1.2). The mice were irradiated and then sensitised on their shaved backs and CH responses were elicited on the ears. The standard laboratory protocol, in which the ears were not

protected during irradiation, resulted in a 31% suppression of CH response by UVB (Figure 3.1.1). Adapting the protocol so that the ears were protected during irradiation increased UVB-induced suppression to 46% (Figure 3.1.2).

4.1.4 The effect of in vivo UVB exposure on antigen-specific DC accessory activity.

Having shown that the exposure of mice to UVB (1440 J/m^2, -48 and -24 hrs) caused the suppression of CH responses (Figures 3.1.1. and 3.1.2), and that epicutaneous treatment with FITC resulted in the accumulation of FITC-bearing DC in the auricular lymph nodes (Figure 3.1.3.b), it was decided to examine the effect of UVB on the ability of DC to present antigen *in vitro* to sensitised LNC. Initial results (Figure 3.1.7) suggested that prior UVB exposure was having no effect on the ability of DC from FITC- or oxazolone-sensitised mice to stimulate antigen proliferative responses from FITC- or oxazolone-sensitised LNC. However, thymidine incorporation was low and the antigen specificity of responses were not convincing. Titrating the proliferative response led to an increase, from $2x10^5$ to $5x10^5$ cells/well, in the numbers of responder LNC used (Figure 3.1.8). Using this adapted protocol, UVB failed to affect the ability of FITC-sensitised DC to act as costimulatory cells on two separate occasions (Figure 3.1.9a and b). This failure of UVB to suppress FITC-specific APC activity was shown over a range of stimulator to responder ratios (Figure 3.1.11a).

Similarly oxazolone-specific proliferative responses were unaffected by UVB. Both oxazolone and FITC sensitised DC could stimulate proliferative responses from oxazolone-sensitised LNC, though FITC DC were poorer accessory cells (Figure 3.1.10). This is in accordance with previous results, where FITC and oxazolone-bearing DC showed a limited antigen non-specific stimulatory capacity (Jones *et al.*1989). However, UVB exposure prior to oxazolone sensitisation failed to influence materially the ability of DC to initiate antigen-specific proliferative responses (Figure 3.1.10).

A number of groups have looked at the effect of UVB on LC function *in vitro* and have shown that UVB has profound effects on the activity of DC. Irradiation of human

epidermal cell suspensions (8-20% LC) and purified LC populations (70-90% LC) with 100-200J/m^2 of UVB *in vitro* significantly inhibited their ability to stimulate primary alloresponses, mitogen-induced proliferation and proliferative responses to recall antigens (Rattis *et al.*1995). Similarly, exposure of murine epidermal cell suspensions to a low dose of UVB (25 J/m^2) inhibits anti-CD3 induced proliferation of T cells (Tang and Udey, 1991) while 250-2000 J/m^2 abrogates EC antigen-specific accessory activity in a dose dependent manner (Stingl *et al.*1981). However, *in vitro* exposure of LC to 100 J/m^2 of UVB is cytotoxic to LC, causing their eventual loss from cultures after 48-72 hrs (Tang and Udey, 1992).

More relevant to this study is work on *in vivo* models. Bucana *et al.* (1994), exposed mice to 400 J/m^2 UVB on 4 consecutive days prior to FITC sensitisation and showed that the accessory activity of enriched DC for FITC specific T cell lines was reduced. The enriched DC cell population contained 10-30% DC following Metrizamide centrifugation, and DC were used at a stimulator: responder cell ratio of 5:1. In the present study, the enriched population contained 50-70% DC on basis of morphology and was used at a ratio of 1:55 or 1:100. It is possible that the suppression of proliferative responses reported by Bucana *et al* could be a result of UVB-induced changes in the large number of non-DC contaminating the stimulator cell population. Alternatively the responding cell population, which was FITC-specific T cell lines in the Bucana *et al* study and LNC stimulated *in vivo* with FITC in our study, could interact differently with DC.

Chronic exposure to 8 kJ/m^2 of UVB 3 times weekly for up to 32 weeks (Alcalay and Kripke, 1991) caused a decrease in the ability of draining LNC populations to transfer CH responses to syngeneic recipients at 4, 12 or 17 weeks. This was ascribed to a reduced capacity of DLN cells to present antigen. However, because of the contrasting protocols, e.g. acute versus chronic and low-dose versus high-dose exposure, it is difficult to compare the results obtained in the chronic study with those presented here. However, the same group have reported similar findings using a much lower dose of

UVB (400 J/m^2 on 4 consecutive days) prior to sensitisation (Okamoto and Kripke, 1987). DLN cells from UVB irradiated and sensitised mice, but not DLN cells from control sensitised mice, induced antigen specific tolerance in unirradiated syngeneic recipients (Okamoto and Kripke, 1987). Although a different UVB exposure protocol was used compared to in the present study, the acute, low-dose exposure protocol allows comparisons to be made. Since DLN cells, exposed to 1600 J/m^2 of UVB over 4 days *in vivo*, transfer antigen-specific tolerance in the Okamoto study, it seems plausible that DLN cells in the present study, that were exposed to 2880 J/m^2 over 2 days, would have the same ability. Therefore, it would have been helpful here to have examined the ability of DLN cells after low-dose immunosuppression to transfer CH responses to syngeneic recipients.

4.1.5 The limitations of this study

In the absence of technical limitations the conclusion from this work is that UVB-induced immunosuppression is not mediated by changes in the function of DC. However, possible weaknesses in the experimental design should be discussed.

4.1.5.1 Is the induction of proliferation an adequate marker of APC function?

The end-point for all functional analyses was the ability of DC to induce proliferative responses, and to find out whether DC from mice that had received an immunosuppressive dose of UVB were less able to stimulate antigen-specific immune responses than DC from unirradiated animals. No difference in the antigen-specific functional activity between DC from control or irradiated mice was found, even though irradiated mice received doses of UVB which caused suppression of CH responses. However, the ability of DC from irradiated mice to induce proliferative response may not equate with their ability to induce functional effector cells. Most cutaneous immune responses are T cell mediated. Therefore, since the description of the CD4$^+$ T cell subsets by Mosmann *et al*, (1986), there has been great interest in whether induction of tolerance following UVB reflects a switch of effector mechanisms from a Th1 to a Th2 response,

manifesting itself in suppressed cutaneous immunity. Th1 responses are critical in the induction of DH responses. Macrophages are the principle effector cells in DH responses and IFN-γ production by Th1 T cells is important in the stimulation of macrophage function. It is thought that other cutaneous responses, for example CH, are mediated via similar Th1 mechanisms.

The evidence for a UVB-induced switch in immune responses is increasing. Initial work showed that exposing LC to UVB caused them to induce antigen-specific unresponsiveness and tolerance in Th1 but not Th2 clones (Simon *et al.*1991). The ability of UVB to suppress Th1 responses *in vivo* was demonstrated (Simon *et al.*1994) using a system similar to the one used in this study. Mice were exposed to UVB (200 J/m^2 on four consecutive days) and were then sensitised with a contact allergen through the irradiated site, immediately following irradiation and again 24 hrs later. UVB treatment was associated with suppressed CH responses and with the reduction of IFN-γ and IL-2 production by draining LNC from irradiated mice *in vitro* (Simon *et al.*1994).

UVB-induced IL-10, produced predominantly by keratinocytes in mice (Enk and Katz, 1992) and by skin infiltrating CD11b macrophages in humans (Kang *et al.*1994) may be important in switching to a Th2 response. Exposure of murine keratinocytes to 200 J/m^2 UVB *in vitro* causes the secretion of IL-10 into supernatants (Rivas and Ullrich, 1992). Protein from irradiated but not from control keratinocyte supernatants blocked IFN-γ production by KLH-specific Th1 cells during culture with APC and antigen (Rivas and Ullrich, 1992). This activity was reversed using an antibody to IL-10. Similarly intravenous injection of supernatants from UVB-irradiated keratinocytes reduced the ability of splenic APC to present antigen to Th1 but not to Th2 T cell clones, an activity which was IL-10 dependent (Ullrich, 1994). The transferable suppressor T cells that are found in the spleen following UVB treatment may be a Th2 effector T cell population. The transfer of suppression of DH responses to syngeneic recipients by splenic cells from irradiated mice was blocked by injecting the recipient mice with neutralising antibody to

IL-10 and IL-4, or by treatment with the Th1-type cytokine IL-12 (Rivas and Ullrich, 1994; Schmitt *et al.* 1995), suggesting that splenic suppressor cells mediate their suppressive activity through the secretion of Th2 cytokines.

Therefore although DC from UVB treated mice are able to induce proliferative responses, it is possible that the quality of effector cells induced by those DC was different from the quality of effector cells induced by DC from unirradiated mice. It would have been interesting therefore to examine the supernatants of sensitised LNC following incubation with DC from irradiated and non-irradiated mice, to see if their cytokine profiles differed.

4.1.5.2 The different requirements of naive and sensitised LNC for professional APC

T cells which express CD45RO, a marker of recent activation, have different APC requirements to naive T cells (Bradley *et al.* 1993). They have less of a requirement for professional APC, such as DC, and their effector function can be activated by non-professional APC in the periphery. Therefore it is plausible that the use of sensitised LNC as responder cells in the functional assays in this study did not provide the most sensitive read out system for subtle changes in APC function. Because the LNC population had 'experienced' the antigen previously it is possible that these cells were less dependent upon costimulatory signals. If this was the case then UVB-induced changes in DC function could have been masked because of the activation state of LNC.

4.1.6 The effect of UVB on the induction of effector cell populations in the lymph nodes

In order to address the possible problems in the experimental protocol highlighted previously, e.g. that the activation state of sensitised LNC was masking possible UVB-induced changes in DC function it was decided to alter the methodology. In order to examine the effect of UVB exposure on the induction of sensitised LNC populations in the DLN, mice were exposed to an immunosuppressive dose of UVB prior to epicutaneous sensitisation and responder LNC were prepared from the DLN 7 days later.

The ability of the responder LNC populations to proliferate in response to DC from FITC or oxazolone sensitised mice was examined *in vitro*. LNC from mice which received UVB treatment prior to sensitisation were equally able to proliferate in an antigen-specific manner during culture with DC (Figure 3.1.12). This suggests that UVB fails to inhibit the induction of antigen specific effector cells following sensitisation. However, like the previous results it is impossible to know whether the LNC populations from irradiated and non-irradiated mice differ functionally. In this respect it would be interesting to examine their cytokine production *in vitro*. Other groups have found differences in the ability of sensitised LNC from irradiated and non-irradiated mice to transfer CH responses to syngeneic recipients, using doses of UVB similar to those used in this thesis. This suggests that there are UVB-induced functional changes in LNC populations following low-dose UVB (Okamoto and Kripke, 1987).

4.1.7 The effect of in vivo UVB exposure on DC activity as accessory cells in MLR

The accessory cell function of DC in a primary allogeneic immune response was examined. It was found that LNC prepared from BALB/c mice sensitised with oxazolone could be stimulated to proliferate in an antigen non-specific manner using lymph node DC enriched from unsensitised C3H/HeN mice. This assay was termed the allergen-activated MLR (AAMLR) (Figure 3.1.15). It was shown that DC isolated from mice that had received a suppressive dose of UVB (1440 J/m^2, -24 and -48 hrs) displayed no loss in their ability to act as accessory cells in the AAMLR (Figure 3.1.16). Similarly exposure to a single higher dose (15 kJ/m2, -24hrs) failed to affect the accessory activity of DC in this assay (Figure 3.1.17).

MLR are commonly used as a measure of DC APC function. The ability of skin-derived DC to act as accessory cells in MLR is increased during their migration to the DLN and LC cultured with keratinocytes or keratinocyte-derived cytokines show similar changes (Romani *et al.*1989). Therefore the effects of UVB on the costimulatory capacity of DC have been assayed by MLR. As mentioned earlier in the Discussion (see section

4.1.4) the exposure of human epidermal cell suspensions (8-20% LC) and purified LC populations (70-90% LC) to 100-200 J/m^2 of UVB *in vitro* significantly inhibits their ability to stimulate primary alloresponses (Rattis *et al.*1995). In another human study enriched human blood DC were exposed to 300, 1000 or 3000 J/m^2 of UVB *in vitro* (Young *et al.*1993), and the two higher doses resulted in a loss of accessory activity in MLR.

Skin biopsies have been used an *in vitro* model of DC migration out of human skin, and to investigate the effect of UVB on DC migration and function. Culture of human skin biopsies resulted in the spontaneous migration of CD1a$^+$ cells out of the skin. Some of these cells had Birbeck granules, a marker for LC, while others expressd markers indicative of dermal DC (Richters *et al.*1994). Exposure of skin biopsies to 75, 150 or 300 J/m^2 of UVB reduced the numbers of LC migrating out of the skin on day 1 and 2 (Richters *et al.*1996). UVB also caused a significant decrease in the ability of the migratory population to induce MLR. The ability of splenic APC from C3H/HeN and BALB/c mice to act as accessory cells in MLR was reduced following exposure to high dose UVB (30 kJ/m^2) (Kitajima and Imamura, 1992).

4.1.8 The effect of in vivo *UVB exposure on DC phenotype*

The expression of three markers (MHC class II, ICAM-1 and B7-2) which are important in the physical interaction and signalling between antigen presenting cells and T lymphocytes was examined. No changes in the percentage of DC expressing these membrane determinants, or the density of expression of markers on DC were found after exposure to UVB (Figure 3.1.27).

4.1.8.1 MHC class II

MHC class II expression is vital for cognate interactions between APC and T cells. Antigen in the form of short peptide chains is presented to CD4$^+$ T cells in the groove of the MHC class II molecule. The TCR on the cell surface of T cells recognises specific peptide chains associated with the MHC class II complex. T cells which

recognise specific antigen on an APC, and which receive the correct costimulatory signals are induced to proliferate and differentiate.

Because of the importance of MHC class II in antigen presentation it was decided to investigate whether the modulation of cutaneous immunity by UVB, was associated with changes in MHC class II expression of DC in the DLN. *In vivo* exposure to immunosuppressive doses of UVB *in vivo* failed to affect the proportion of DC expressing MHC class II or the intensity of expression (Table 3.1.4, Figures 3.1.25 and 3.1.27). This failure of UVB to affect MHC class II expression agrees with a number of other studies. Low-dose UVB (100 J/m^2) inhibited culture-induced up-regulation of ICAM-1, but not MHC class II, on murine LC (Tang and Udey, 1991). Similarly the up-regulation of MHC class II expression on human LC during culture was unaffected by exposure to 100 J/m^2 UVB (Rattis *et al.*1995). Higher doses of UVB (1000 and 3000 J/m^2) caused a slight reduction in low-intensity but not high-intensity MHC class II expression on human blood DC (Young *et al.*1993). In the human skin equivalent model (Richters *et al.*1996), UVB exposure (75-300 J/m^2, actual dose not stated) did not affect the percentage of MHC class II$^+$ cells migrating out of the skin biopsies.

The work that is closest in terms of methodology to that undertaken for this thesis is an *in vivo* study in which the phenotype of cells in lymph nodes draining UVB exposed and contact sensitised skin were examined (Bucana *et al.*1994). Mice were exposed to 400 J/m^2 of UVB on 4 consecutive days and were sensitised through the irradiated site soon after the last irradiation. In agreement with previous studies by the same group DLN cells from UVB irradiated and sensitised mice, but not DLN cells from control sensitised mice, induced antigen specific tolerance in unirradiated syngeneic recipients (Okamoto and Kripke, 1987). The percentage of MHC class II$^+$ FITC$^+$ DC in the draining lymph nodes of mice which were untreated or exposed to UVB prior to FITC sensitisation was assessed. Prior treatment with UVB caused a slight increase from

36-41% of MHC class II^+ $FITC^+$ DC. In the present study, UVB failed to affect the percentage of DC within the DLN that expressed MHC class II.

4.1.8.2 ICAM-1

ICAM-1 expression is important for antigen presentation as the interaction between this molecule and LFA-1 is involved in the binding of APC and T cells and there is also evidence for activation signals being induced by ICAM-1/LFA-1 interactions. The importance of ICAM-1 expression has been demonstrated in mutant mice that have low levels of ICAM-1 expression. Cells deficient in ICAM-1 showed reduced accessory activity which was restored by the reintroduction of a functional ICAM-1 gene (Dang *et al.*1990). The up-regulation of ICAM-1, and other surface molecules, on lymph node DC compared with epidermal LC (Cumberbatch *et al.*1992) corresponds with increased APC activity. Because ICAM-1 is a constituent of APC activity, a number of groups have investigated the effect of UVB on expression of ICAM-1 on LC.

Exposure of murine LC to UVB *in vitro* caused a reduction in ICAM-1 expression. Freshly isolated murine LC were untreated or were exposed to 100 J/m^2 of UVB, prior to culture for 24, 48 or 72 hrs (Tang and Udey, 1992). Exposure to UVB reduced the recovery of LC from culture at all timepoints and selectively inhibited the up-regulation of ICAM-1 but not MHC class II expression on LC (Tang and Udey, 1992). The effect of UVB on ICAM-1 expression by enriched human blood DC has also been examined using higher doses of UVB. Exposure to 1000 or 3000 J/m^2 of *UVB in vitro* caused a dose-dependent inhibition of ICAM-1 up-regulation on DC during culture. The inhibition of ICAM-1 up-regulation during culture may reflect DNA damage. People who have xeroderma pigmentosum (XP) exhibit deficient DNA repair, and fibroblasts from these individuals are more susceptible to UVB-induced suppression of IFN-γ induced ICAM-1 expression than fibroblasts from normal individuals (Krutman *et al.*1994). This may help to explain the differences seen following *in vitro* versus *in vivo* UVB exposure, since *in vitro* UVB exposure is likely to result in more DNA damage to LC, than occurs

following UVB-exposure *in vivo*. Irradiation with low doses of UVB light *in vitro* causes cytotoxicity, particularly to LC which are very sensitive. Exposure to 100 J/m^2 of UVB reduced the recovery of LC in 72 hr EC cultures from around 33% to 3% (Tang and Udey, 1992). Therefore, the results obtained *in vitro* may reflect responses to cellular injury. When animals are irradiated, UVB loses much of its energy as it penetrates through the layers of stratum corneum. In addition since UVB causes the loss of LC from the skin, via mediators such as TNF-α (Cumberbatch *et al.*1994), it is plausible that LC migrate out of the skin before they accumulate excessive DNA damage.

4.1.8.3 B7-2

B7-1 and B7-2 (CD86) are members of the immunoglobulin supergene family, the genes of which have been cloned (Freeman *et al.*1993; Azuma *et al.*1993). The expression of B7-1 and B7-2 on DC populations has been described in detail in section 1.5.2, and Table 1.2 provides a summary of changes in the expression of these molecules during LC culture. Briefly, B7-1 is not expressed on murine or human LC (Table 1.1), but is induced on cultured LC (Table 1.2). B7-2 is expressed at low levels on murine LC (Table 1.1) and its expression is upregulated on these cells during culture (Table 1.2). B7-1 is induced to lower levels during culture than B7-2, which is expressed at very high levels following culture of LC (Larsen *et al.*1992; Symington *et al.*1993; Inaba *et al.*1994).

B7-1 and B7-2 function by binding to their ligands on T cells, CD28 and CTLA-4. It is thought that costimulatory signals, such as those mediated by B7-1 and B7-2, are vital for the induction of effector cells. T cells binding to APC via their TCR to MHC/antigen complexes, will only be induced to differentiate and proliferate if they receive the appropriate second signals, mediated by costimulatory molecules. In the absence of these signals, the outcome is T cell anergy or cell death (Nickoloff and Turka, 1994). The relative contribution of B7-1 and B7-2 to DC APC activity has been discussed in section 1.5.2. In the same section evidence was presented that signals mediated

through B7-1 may induce Th1 type immune responses, while B7-2 signals mediate Th2 responses (Kuchroo *et al.*1995).

In this study, it was found that *in vivo* UVB exposure failed to alter the expression of B7-2 on DC isolated from lymph nodes draining the site of exposure (Table 3.1.5 and Figure 3.1.26). This contrasts with results from other systems, where UVB has been shown to alter the expression of this molecule. Murine epidermal LC were exposed to 100 or 200 J/m^2 of UVB *in vitro* (Weiss *et al.*1995). Both doses inhibited the ability of LC to up-regulate B7-1 or B7-2 during culture. This reduction in costimulatory molecule expression by LC affected their function as APC in mixed epidermal cell lymphocyte reactions (MECLR) (Weiss *et al.*1995). EC suspensions which were irradiated with 200 J/m^2 of UVB prior to their use as accessory cells in MECLR were poor stimulator cells compared with untreated EC. However, the addition of an anti-CD28 monoclonal antibody which mimicked costimulatory signals by crosslinking CD28, restored the accessory activity of the irradiated EC. Exposure of enriched human blood DC to higher doses of UVB (1000 and 3000 J/m^2) *in vitro* caused the dose-dependent inhibition of B7-1 up-regulation during a MLR (Young *et al.*1993). In the skin equivalent model (Richters *et al.*1996) exposure to UVB induced a small reduction in the percentage of migrating cells which expressed B7-1 or B7-2, though the reduction was generally less than 10%. The expression of B7-1 and B7-2 on 'large cells' in the migrated population was analysed by indirect labelling using the alkaline phosphatase method and direct microscopic examination. Staining with CD1a and CD14 showed the population of large cells to be comprised of both LC and macrophages. Since the numbers of CD1a$^+$ LC in the migrating population were reduced by around 10% following irradiation, it seems likely that the reduction of B7-2 following irradiation reflects the increased proportion of macrophages within the migrating population.

4.1.9 Summary of the effect of in vivo *UVB-exposure on DC function and phenotype*

On the basis of this study it is impossible to explain the immunosuppressive effect of UVB *in vivo* through changes induced in the functional activity of DC. However as discussed earlier (section 4.1.5.1), by examining only proliferative responses in this study, more subtle changes in DC induced by UVB may have been ignored. There is evidence that local exposure to UVB causes the preferential production of Th2 type cytokines by DLN cells (Araneo *et al.*1989; Simon *et al.*1994). Irradiation may alter the DC arriving in the DLN, or alternatively change the lymph node microenvironment, causing the selective activation of a particular T cell subset. Therefore, measuring the ability of DC to stimulate proliferative responses may be of less importance than examining the functional activity of the effector T cells induced to proliferate by the DC. In addition, by testing the function of DC *in vitro*, the effect of the lymph node microenvironment is ignored and the results may not reflect the antigen-presenting activity of DC *in vivo*.

It is believed that many of the DC found within the skin-draining lymph nodes derive from epidermal LC. Therefore it is interesting that the susceptibility of LC to UVB *in vitro* is not mirrored by similar changes in lymph node DC following exposure to UVB *in vivo*. There may be a number of explanations for this observation. In the *in vitro* studies, LC in suspension were exposed directly to UVB at doses that are cytotoxic. Therefore, the results obtained *in vitro* may reflect responses to cellular injury. When animals are irradiated, UVB loses much of its energy as it penetrates through the layers of stratum corneum. Therefore, in addition to the direct damage to DNA that UVB can cause, UVB induced mediators may be important, especially in the immunosuppression following low doses of UVB. Secondly, *in vitro* and *in vivo* studies could be examining different populations of APC. Some groups support the hypothesis that UVB acts to 'trap' LC transiently in the epidermis via a TNF-α dependent mechanism (Streilein, 1993). Results in the human skin equivalent model where exposure to UVB

results in a minor drop, around 10%, in the numbers LC migrating out of the skin has been used to support this theory (Richters *et al.*1996). If this were the case then DC accumulating in the DLN following UVB radiation might not be expected to show UVB-induced changes, because the alterations in the kinetics of DC accumulation would result in the accumulation of DC that had managed to avoid UVB-induced LC 'trapping' in the skin. However UVB has been shown to reduce LC numbers in the skin (El-Ghorr *et al.*1994) and cause the accumulation of DC in the DLN (Moodycliffe *et al.*1992), and there is good evidence for TNF-α being an important mediator of UVB-induced LC migration (Cumberbatch *et al.*1994) and DC accumulation (Cumberbatch and Kimber, 1995; Moodycliffe *et al.*1994). There is a greater breadth of evidence for UVB-induced LC migration than LC 'trapping'. Therefore it is likely that similar populations of cells, e.g. LC derived DC, are being examined in the *in vitro* and *in vivo* models. The only difference may be in the dose of UVB that LC are being exposed to in each case.

As well as the lack of a UVB-induced suppression of effector function in DC *in vivo*, it seems that UVB also failed to affect the phenotype of these cells. It is not possible therefore on the basis of the expression of Ia, ICAM-1 or B7-2 to relate the immunosuppressive properties of UVB *in vivo* to alterations in DC phenotype. The results presented in this thesis contradict studies of UVB exposure *in vitro*, where UVB altered ICAM-1 and B7-2 expression. However, the difference between *in vivo* and *in vitro* UVB exposure are manifold and have been described previously. *In vivo* the mechanical barrier of the skin quickly reduces the energy of UVB as it penetrates into the epidermis and, together with the migratory potential of LC, is likely to protect LC from serious UVB-induced DNA damage. This cannot be said for *in vitro* studies, except perhaps for the skin equivalent model. *In vitro* UVB exposure, even following the low doses used in most of the studies, is likely to result in DNA damage and cellular cytotoxicity.

However, common to all *in vivo* studies involving DC are problems with identifying the derivation of DC in the DLN. Therefore, it could be argued that the DC populations examined in this study do not represent cells which have been exposed to UVB in the skin, but are instead derived from other sources. Earlier in this discussion, the work of other groups (Macatonia *et al.*1987; Kripke *et al.*1990) which suggest that the majority of DC draining sensitised sites are of cutaneous origin. However, the techniques used in these studies are not universally endorsed and there is some controversy over the interpretation of the results. These arguments centre on the ability of FITC to migrate to the DLN without first binding to LC, or other migratory APC populations in the skin. However, it seems likely that at least a proportion of FITC-bearing cells which accumulate in DLN following epicutaneous application of this allergen are skin derived, particularly those DC which express high intensity fluorescence (Macatonia *et al.*1987). Therefore, if UVB exposure was altering the expression of MHC class II, ICAM-1 or B7-2 on these cells, then it seems likely that these changes would have been seen within enriched DC populations from DLN.

Similarly if the immunosuppression following UVB was mediated, as has been suggested, by the accumulation of non-LC derived DC in the DLN, changes in the expression of MHC class II, ICAM-1 and B7-2 would be expected. However there are possible weaknesses in methodology associated with this observation. Firstly, the analysis of surface expression was to a great extent limited to cells with the size and scatter characteristics of DC within enriched DC populations. In most experiments DC-depleted populations were also analysed and no differences were observed in the expression of the three markers. However, because for the most part analyses were restricted to large granular cells it is possible that changes in the other populations within enriched and depleted populations went unobserved. The second weakness is related and concerns the use of indirect single colour fluorescent staining. Without employing dual or multi-colour staining, it is difficult to definitively identify cell populations, and thus,

changes within DLN cell populations may not have been detected. However, the remit for the study presented in this thesis was the examination of the role of DC in UVB-induced immunosuppresson. From the evidence presented here it seems unlikely that changes in MHC class II, ICAM-1 or B7-2 on DC following UVB exposure is responsible for the immunosuppression seen after UVB exposure *in vivo*.

The number of molecules that could be examined in this study was limited. The molecules that were chosen had been shown to be important in antigen presentation. Due to their importance in antigen-presentation, it was felt that these molecules could be a possible target for UVB-induced alterations in APC activity. UVB exposure *in vivo* did not alter the expression of the three surface molecules examined here. However, it cannot be concluded that UVB will not influence other surface molecules, such as B7-1 (CD80) or heat stable antigen (HSA) both of which have costimulatory function (Larsen *et al.*1992; Liu *et al.*1992).

The marked changes in LC phenotype and function induced by *in vitro* UVB exposure are not apparent in DC populations in lymph nodes draining the site of exposure to UVB *in vivo*. *In vitro* UVB results in a substantial decrease in LC accessory cell activity and a reduction in the culture induced up-regulation of certain surface molecules. If these UVB-induced changes which are seen *in vitro* are mirrored by DC migrating to the DLN after irradiation *in vivo*, it would be possible to speculate that these DC would fail to induce appropriate effector T cell responses. However, this seems not to be the case since DC which accumulate in the DLN of C3H/HeN mice following irradiation *in vivo* are identical in function and phenotype to DC from control mice. Caution must be exercised therefore when using *in vitro* data to investigate the complex responses seen *in vivo* after exposure to UVB.

4.2 IL-6 in the draining lymph nodes and skin of contact sensitised mice.

4.2.1 Introduction

The initial interest in this area arose from three observations which suggested that IL-6 production in the DLN had an important role in the induction of effector cell responses to contact allergens. These observations were discussed in the introduction to the IL-6 results section (3.2.1) but for clarity will be restated here. The first observation came from examining proliferative responses in the DLN during the induction phase of contact sensitisation. The local lymph node assay is a procedure used to identify the sensitising potential of chemicals (Scholes *et al.*1992). In this assay the potency of the sensitising agent is measured as a function of the vigour of the primary T lymphocyte proliferative response in lymph nodes draining the site of epicutaneous application, with proliferative activity increasing with the strength of the sensitising chemical. The second observation, this time in BALB/c mice, suggested that IL-6 production in the local lymph node was important in the induction of proliferative responses (Hope *et al.*1994) and that in fact, the level of IL-6 production by LNC draining the sensitised site, could be used instead of proliferative activity, as an end-point for the local lymph node assay (Dearman *et al.*1993). Finally, it was observed in BALB/c mice that DC provided the main source of IL-6 within sensitised lymph node populations (Hope *et al.*1995, Cumberbatch *et al.*1996). The evidence for DC being the main source of IL-6 in sensitised lymph nodes will be discussed in more detail later in this section. Pulling together these studies and others, a model of the induction of contact hypersensitivity can be proposed. The migration of antigen-bearing LC from the skin, their maturation into potent APC, and their accumulation in the DLN is well documented and has been discussed in the introduction. In the lymph node DC present antigen to naive antigen-responsive T lymphocytes, inducing them to proliferate and differentiate. This process is complex involving physical interaction and signalling between cells. The role of MHC class II,

ICAM-1 and B7-2 in this process has been discussed previously (sections 4.1.8.1, 4.1.8.2 and 4.1.8.3). Since IL-6 production by DC correlates strongly with proliferative activity in the lymph node, it seems likely that soluble signalling is also important in antigen presentation. IL-1 and IL-6 have particular importance in the early stages of T lymphocyte activation because of their ability to induce IL-2 production and IL-2 responsiveness respectively (Van Snick, 1990). The production of IL-2, and the expression of the IL-2 receptors by T lymphocytes, determines their ability to proliferate and mature/differentiate into an antigen-specific effector cell. In summary, IL-6 production by lymph node DC during the induction phase of contact sensitisation is likely to be important in the generation of effector T lymphocytes.

The evidence for the association of IL-6 production and proliferative activity in DLN during the induction of contact sensitivity was gained in models using the BALB/c strain of mice. This strain is UVB-resistant in both local and systemic models of UVB-induced immunosuppression (Streilein and Bergstresser, 1988; Noonan and Hoffman, 1994), as discussed in the introduction (section 1.7.5). Having access to both UVB-resistant (BALB/c) and susceptible (C3H/HeN) strains of mice, whose CH responses were either unaffected, or modulated by UVB exposure, it was of interest to examine the production of IL-6 by LNC in these strains and to determine whether the UVB-induced suppression of CH responses in C3H/HeN mice was associated with changes in IL-6 production within lymph nodes of irradiated mice.

4.2.2 Strain differences in UVB-induced immunosuppression

To confirm previous observations of the respective susceptibility and resistance of C3H/HeN and BALB/c mice to UVB (Streilein and Bergstresser, 1988, Noonan and Hoffman, 1994), both strains were exposed to UVB (1440 J/m^2, -48, -24 hrs) prior to sensitisation with oxazolone. Six days later CH responses were elicited by ear-painting with the same chemical. In Figure 3.2.1 the ears of mice were uncovered during UVB irradiation while in Figure 3.2.2 the ears were protected. The change in protocol was

implemented in response to UVB-induced damage to the ears of BALB/c mice which had not been observed previously with C3H/HeN mice. Because the ear damage, which involved swelling and flaking of the ears, corresponded with the elictation of CH responses on day 6 following sensitisation, it was felt that it could be detrimental to the end-point of the experiment. Therefore the experiment was repeated with the ears of mice protected during irradiation, and the results of this experiment are shown in Figure 3.2.2.

In Figure 3.2.1a exposure of C3H/HeN mice to 1440 J/m^2 of UVB 48 and 24 hours prior to sensitisation caused a 31% suppression of CH responses to oxazolone. Exposure of BALB/c mice to the same dose of UVB failed to suppress CH responses and instead caused a slight increase in allergen-induced ear swelling (Figure 3.2.1b). The intensity of CH responses was similar in both strains with maximum increase in ear swelling, following sensitisation being around 0.15 mm. However, because UVB was causing actual physical damage to the ears of BALB/c, but not C3H/HeN mice, it makes it impossible to compare CH responses between groups. Therefore, the experiment was repeated with the mice anaesthetised, and their ears protected, during irradiation. All the groups of mice were anaesthetised, whether they received UVB or not, to control for possible effects of the anaesthetic. Figure 3.2.2a shows the results. Protecting the ears of C3H/HeN mice does not seem to have affected the suppression of CH response compared with Figure 3.2.1a. In both experiments the mean allergen-induced ear swelling of UVB treated C3H/HeN mice is around 0.11 mm. However, in the second experiment (Figure 3.2.2a) the positive control for C3H/HeN mice is higher than the positive control in the first C3H/HeN experiment (Figure 3.2.1a), around 0.18 and 0.15 mm respectively. Due to the higher positive control, the CH response of the C3H/HeN mice was suppressed by 46% in Figure 3.2.2a. This level of suppression has been reported previously for C3H/HeN mice following an identical protocol of UVB-exposure (Moodycliffe *et al.*1994). Using the same protocol with anaethesitisation and ear protection, the suppression of CH responses in BALB/c mice was examined (Figure

3.2.2b). It can be seen that in the absence of the UVB-induced ear damage which was found in the first experiment (Figure 3.2.1), UVB exposure of BALB/c mice still failed to suppress CH responses (Figure 3.2.2b). This confirms previous studies of CH responses, which were not suppressed in BALB/c mice following exposure to doses of UVB which caused the suppression of CH in C3H/HeN mice (Streilein and Bergstresser, 1988; Noonan and Hoffman, 1994). However, it is clear that the CH response in BALB/c mice is particularly low, with the mean increase in ear thickness being around 0.05mm in both the positive and UVB-exposed groups (Figure 3.2.2b). The reason for this is uncertain, and, had it been possible, it would have been worthwhile repeating this experiment to confirm the findings following the induction of comparable CH responses in both C3H/HeN and BALB/c mice. However, good evidence for the strain difference between C3H/HeN and BALB/c exists (Streilein and Bergstresser, 1988; Noonan and Hoffman, 1994), and this evidence has been supported, though due to the limited nature of the experiments completed, not conclusively, by results presented here.

4.2.3 IL-6 production by draining lymph node cells

Since the ability of UVB to suppress CH responses was first investigated (Toews *et al.* 1980) alterations in LC and LC-derived DC, have been central to a number of hypotheses presented to explain the phenomenon. These have been covered in the introduction (section 1.8.1.1). Because C3H/HeN and BALB/c mice show differing responsiveness to UVB-induced immunosuppression, and IL-6 is important in the induction of CH responses in BALB/c mice, it was of interest to determine whether UVB affected the production of this cytokine in C3H/HeN and BALB/c mice. However, in initial experiments examining IL-6 production in both strains, a marked and highly reproducible pattern of IL-6 production emerged. Following sensitisation, proliferative responses were induced to a similar extent in both strains, though proliferative responses between individual experiments were highly variable (Table 3.2.1). There was, however, a marked strain difference with respect to the ability of LNC to secrete IL-6 after skin

sensitisation. LNC from BALB/c mice produced high levels of IL-6 *in vitro* with IL-6 levels peaking at around 8 ng/ml 3 days after sensitisation (Table 3.2.1). This is similar to levels found following oxazolone sensitisation of BALB/c mice in a previous study (Hope *et al.*1994). In contrast, draining LNC from C3H/HeN mice secreted low or undetectable levels of IL-6 when sensitised identically (Table 3.2.1). So unlike in BALB/c mice where LNC proliferation is closely associated with IL-6 production (Hope *et al.*1994), in C3H/HeN mice LNC proliferative activity is not associated with IL-6 expression. The ability of sensitised LNC from C3H/HeN mice to proliferate in the absence, or in the presence of very low levels of IL-6 activity, suggest either that IL-6 availability is not an absolute requirement for LNC activation, or that the low concentrations of IL-6 secreted in culture by C3H/HeN LNC give sufficient costimulation for T cell activation.

4.2.4 IL-6 production by sensitised LNC in other UVB-susceptible and resistant mice.

Having shown a marked difference in the ability of sensitised LNC from C3H/HeN and BALB/c mice to produce IL-6 following sensitisation, it was decided to examine further UVB susceptible and resistant strains to determine whether the differential ability of LNC to produce high concentrations of IL-6 following sensitisation could provide a marker for the responsiveness of mice to the immunosuppressive effects of UVB.

In order to investigate this issue, three strains of mice were chosen, namely C57BL/6 (UVB-susceptible), DBA/2 (UVB susceptible) and AKR (UVB resistant) mice, and IL-6 production and proliferative activity by LNC from each strain following sensitisation was examined. Sensitisation induced proliferative responses by LNC in all the strains tested (Figures 3.2.4, 3.2.5, 3.2.6). The magnitude of LNC proliferative responses of C57BL/6 and AKR mice were comparable, although there were differences in kinetics, with peak proliferation on day 2 and day 3 respectively (Figures 3.2.4 and 3.2.6). The proliferative activity of LNC from DBA/2 was higher (Figure 3.2.5), and

displayed similar kinetics to the AKR strain (Figure 3.2.6), with peak activity on day 3 following sensitisation. However, none of the strains produced appreciable IL-6 activity, and so the results do not support the absence of IL-6 activity in lymph nodes following sensitisation, as being a marker of UVB susceptibility. Table 3.2.2 summarises the conclusions from the local and systemic models (Noonan and Hoffman, 1994; Streilein and Bergstresser, 1988) and the proliferative and IL-6 data from section 3.2.3.

However, there are a number of problems with this study which should be discussed. These strains were classified as UVB-susceptible and resistant following the work of Noonan and Hoffman, (1994), that characterised inbred mouse strains susceptibility to UVB (low, intermediate and high) over a range of doses. C57BL/6 mice were highly susceptible, DBA/2 and C3H/HeN mice were intermediately susceptible and AKR and BALB/c mice showed low susceptibility (Noonan and Hoffman, 1994). C57BL/6 mice were available from the Medical Microbiology Transgenic Unit, Edinburgh University, while DBA/2 and AKR mice were only available commercially. Being unable to get the numbers of DBA/2 and AKR mice needed to examine their responsiveness to UVB in the acute model used generally in this thesis, meant relying on the Noonan and Hoffman study for the classification of all the strains. However, there are some problems which have become obvious following the use of this approach. Firstly, the Noonan and Hoffman study uses a systemic model of UVB-induced immunosuppression. The backs of mice were exposed to UVB over a dose range of approximately 1.2-20 kJ/m^2, which includes the doses used in routinely in the present study (2.88 kJ/m^2 total). However, sensitisation was through a non-irradiated site 3 days after exposure to UVB, rather than 24 hrs later on the irradiated site that has been used in this study. Therefore the Noonan and Hoffman study examined systemic immunosuppression while the protocol used in this study examined the suppression of local cutaneous immunity. However, since Noonan and Hoffman showed BALB/c and C3H/HeN mice to have the same phenotype of UVB susceptibility in the systemic model,

as had been shown in local models (Streilein and Bergstresser, 1988) it was decided to use the systemic classification for C57BL/6, DBA/2 and AKR mice. While there is some controversy in the literature about the status of these strains, there is agreement over C3H/HeN, BALB/c and C57BL/6 mice. DBA/2 mice are however classified as UVB susceptible in the systemic model (Noonan and Hoffman, 1994) and UVB resistant in the local model (Streilein and Bergstresser, 1988), while AKR mice have only been studied in the systemic model. Because of these problems this study has not definitively addressed whether susceptibility to the local immunosuppression which follows UVB is associated with an inability to produce IL-6 in the DLN.

Therefore the issue of whether IL-6 production, or lack of it, in the DLN is associated with the resistance/susceptibility of inbred mice strains to UVB is still open. However it would be interesting to speculate on the mechanisms by which IL-6 could affect UVB responsiveness. Although IL-6 is important in the initial stages of antigen presentation, LNC from mice that produce low levels of IL-6 following sensitisation show comparable proliferative activity. Therefore it seems unlikely that the low levels of IL-6 production are inhibiting the ability of DC to present antigen, as measured by the induction of proliferative responses. This conclusion is fairly obvious especially as C3H/HeN mice, which produce low levels of IL-6 following sensitisation, respond normally to epicutaneous sensitisation. Therefore in the absence of UVB, the inability of LNC to produce high levels of IL-6 following sensitisation, does not appear to be detrimental to the induction of an immune response. This suggests that either IL-6 has activity at the levels produced by C3H/HeN mice following sensitisation, or that there is redundancy in IL-6 activity with another cytokine, either alone or in combination, being able to mediate IL-6 activity.

However, the strain differences observed in UVB-induced immunosuppression are interesting and a number of hypotheses have been put forward to explain the effect of UVB on the cutaneous immune system, and the resistance of some mice strains and

humans to the suppressive effects of irradiation (section 1.7.5). Because of problems with the characterisation of the inbred strains used in this study, strain differences in IL-6 production cannot be proposed as a mediator of UVB susceptibility or suppression. However, since such a dramatic strain difference exists it would be interesting to speculate on functional differences in the effector cells induced in such polarised DLN microenvironments.

4.2.5 IFN-γ production by LNC during the induction of contact sensitisation.

In order to determine whether the inability of C3H/HeN LNC was specific for IL-6 or reflected a more generalised inability of LNC to secrete cytokines, the production of IFN-γ by LNC from both C3H/HeN and BALB/c mice following sensitisation was examined. The results from three separate experiments are shown in Table 3.2.3, where it can be seen that both proliferative activity and IFN-γ levels were similar for C3H/HeN and BALB/c mice. This suggests that sensitisation is inducing similar levels of T lymphocyte activation in both strains. Although there was no significant difference in the IFN-γ levels between strains, the higher levels of production in BALB/c mice may reflect slightly higher level of activation in the lymph nodes, though a comparison of the proliferative activity of both strains does not support this.

4.2.6 DC accumulation in the local lymph nodes following sensitisation.

DC are the main source of IL-6 within BALB/c LNC populations. Complement-depletion of DC from oxazolone-sensitised LNC suspensions reduces IL-6 activity by more than 75% but does not affect proliferative responses (Hope *et al.* 1995). If the same method is used to deplete T cells, the proliferation but not the IL-6 production by LNC *in vitro* is affected. Because DC are the main source of IL-6 within lymph nodes, the number of DC that accumulated in the DLN of both strains following sensitisation was examined. As stated in section 3.2.5 DC numbers were similar in both strains. Vehicle sensitised C3H/HeN mice had a mean of 2759 DC per lymph node which rose to

23479 DC per lymph node 18 hrs after sensitisation. Similarly BALB/c mice had means of 2127 and 22920 DC per lymph node, respectively, following epicutaneous application with vehicle and oxazolone. Therefore the lack of IL-6 production in C3H/HeN mice does not reflect a paucity of DC in the DLN.

4.2.7 Intracellular IL-6 activity in LNC from C3H/HeN and BALB/c mice

LNC from C3H/HeN and BALB/c mice were sonicated to examine strain differences in the intracellular levels of IL-6. Sonicating LNC immediately after removal from C3H/HeN and BALB/c mice sensitised with oxazolone 3 days earlier, caused the release of appreciable quantities of IL-6 into supernatants, while sonication of LNC from mice treated with the vehicle alone resulted in less IL-6 activity in the supernatant. As LNC from both strains contain detectable IL-6 protein, this suggests that both are capable of translating IL-6 protein (Figure 3.2.8). The inability of oxazolone-sensitised LNC from C3H/HeN mice to secrete IL-6 *in vitro* may be associated with a lack of IL-6 production, or may reflect a failure in the ability of cells to secrete IL-6. To investigate this, oxazolone-sensitised LNC from C3H/HeN and BALB/c mice were cultured for 24 hrs and the concentration of IL-6 in the supernatants and sonicates was measured. The inability of C3H/HeN mice to secrete IL-6 in culture was not associated with an accumulation of intracellular IL-6 (section 3.2.6). Therefore it is likely that the low levels of IL-6 produced by C3H/HeN LNC reflect a lack of IL-6 production by LNC, rather than a failure in IL-6 secretion.

4.2.8 UVB-induced DC accumulation and IL-6 production

In agreement with previous studies (Moodycliffe *et al.* 1992), we found UVB-induced accumulation of DC in the local DLN of C3H/HeN mice and in addition recorded comparable numbers of DC in the DLN of BALB/c mice following UVB (section 3.2.7). However, at least in the BALB/c strain, these DC differ substantially from those which migrate to the DLN after contact sensitisation. While LNC from sensitised BALB/c mice

produce high levels of IL-6 and T cell derived IFN-γ, LNC from UVB treated mice fail to produce either cytokine (data not shown for IFN-γ). This suggests that DC migration is not in itself sufficient for all aspects of DC maturation and that another, possibly antigen dependent, signal is required for IL-6 production by DC and the induction of IFN-γ production by T cells.

4.2.9 IL-6 production in the skin of C3H/HeN and BALB/c mice.

The kinetics of IL-6 production in the skin after treatment with oxazolone was also investigated. Previous studies using contact allergens and irritants have shown that IL-6 is induced by contact allergens but not irritants, when these chemicals are used at doses which cause comparable inflammatory responses (Holliday *et al.* 1996). Similar levels of IL-6 were produced constitutively in the skin of both C3H/HeN and BALB/c mice (Figure 3.2.9). In both strains oxazolone treatment induced significant increases in IL-6 production peaking 4 hours after sensitisation (Figures 3.2.9 and 3.2.10). Therefore the lack of IL-6 secretion by sensitised LNC from C3H/HeN mice does not reflect a systemic inability to produce the cytokine. IL-6 is produced constitutively in the skin of BALB/c mice by LC (Cumberbatch *et al.* 1996). Keratinocytes do not produce the cytokine consititutively but are induced to produce it following sensitisation (Shreiber *et al.* 1992; Bos and Kapsenberg, 1993). This study has not addressed whether LC in the skin of C3H/HeN mice have the ability to produce IL-6, although the fact that C3H/HeN and BALB/c mice display constitutive cutaneous IL-6 activity is suggestive that they do.

4.3 The effect of UVB on IL-6 production in the skin

UVB is a potent stimulator of cutaneous cytokine production, up-regulating the expression of TNF-α, IL-10 and IL-6 (Köck *et al.*1990; Grewe *et al.*1995; Kirnbauer *et al.*1991). Since C3H/HeN and BALB/c mice display differing responsiveness to UVB, it was of interest to see if this was reflected in differing effects of UVB on cutaneous cytokine induction between strains.

The first experiment looked promising with UVB causing a significant increase in untreated, AOO- and oxazolone-treated skin of C3H/HeN but not BALB/c mice (3.2.11). However, when repeated there was no inter-strain differences in cutaneous IL-6 activity following UVB (Figure 3.2.12). In this experiment the peak IL-6 levels were higher than had been seen in previous experiments, with over 100 ng/g of tissue compared with the usual 50 ng/g of tissue. However previous studies examining the induction of cutaneous IL-6 by oxazolone in BALB/c mice have reported peak levels around 90 ng/g of tissue (Holliday *et al.* 1996).

As discussed in section 3.2.9 there were differences in the protocol used to quantify epidermal IL-6 activity which may explain the difference in the level of IL-6 detected, particularly between the experiments examining the effect of UVB on cutaneous IL-6 production (Figures 3.2.11 and 3.2.12). The differences between the protocols used are discussed in section 3.2.9. The main effects of the changed protocol used for the experiments shown in Figures 3.2.10 and 3.2.12 were to reduce processing time and the number of freeze thaw cycles and to increase the accuracy of tissue weights, and so may explain the increased levels of IL-6 seen in these experiments.

4.3.1 Summary

The initial aim of this study was to examine strain differences in the effect of UVB on IL-6 production, but the finding that C3H/HeN mice produced little or no IL-6 following sensitization forced a change of thinking. That there was such a dramatic difference in IL-6 production between the two strains in IL-6 production, which in

BALB/c mice at least, was strongly associated with primary T lymphocyte proliferative responses and hence, from the findings of the local lymph node assay, to the strength of CH responses, was of great interest. LNC proliferative responses and IFN-γ production by LNC from C3H/HeN mice were as vigorous as those in BALB/c mice, suggesting that the low-level of IL-6 production was not affecting the induction of effector cell populations under normal circumstances. Interestingly, UVB-induced DC accumulation in the lymph nodes of BALB/c was not associated with IL-6 production, suggesting that the induction of DC migration does not necessarily result in all aspects of DC maturation at least in terms of IL-6 production.

Having started with the aim of determining the ability of UVB to modulate IL-6 production, the finding that C3H/HeN and BALB/c mice displayed such radical differences in the production of this cytokine, led to the speculation that the ability to produce this cytokine in the lymph node during the induction of CH may a marker of resistance to UVB-induced immunosuppression and that conversely, a lack of IL-6 production in the DLN following sensitization could correlate with susceptibility to UVB. To attempt to answer this a further two UVB-susceptible and one UVB resistant strain were tested. In all strains sensitisation induced proliferative activity but little or no IL-6 production. These results suggest that IL-6 production by LNC does not correlate in any way with the responsiveness of inbred mouse strains to UVB-induced immunosuppression. However the problems in classifying the UVB susceptibility of the strains means that no definitive conclusions can be made.

The low levels of IL-6 produced by LNC from C3H/HeN mice did not reflect low numbers of DC accumulating in the lymph nodes of these mice following sensitisation. In addition, sonication experiments suggested that LNC from C3H/HeN mice show lower levels of IL-6 production than those from BALB/c mice.

The low levels of IL-6 produced by LNC from C3H/HeN mice did not reflect reduced production of IL-6 systemically. C3H/HeN mice produced comparable or higher

levels of cutaneous IL-6 than BALB/c mice following oxazolone sensitisation. Keratinocytes are likely to form the main source of IL-6 in the skin following sensitisation, but do not produce the cytokine constitutively. The source of IL-6 production in the skin was not examined, though the constitutive IL-6 production in the skin of C3H/HeN mice indicates that a non-keratinocyte source, probably LC, have the ability to produce IL-6.

Although this study failed to show a conclusive link between IL-6 production and UVB susceptibility, it did however produce a number of interesting findings. The possible functional differences following the induction of effector cell populations, in the presence of high and low levels of IL-6, in strains of varying UV susceptibility are unknown at present.

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Publications arising from this thesis

Papers:

1. Lappin, M. B., El Ghorr, A.A., Kimber, I., and Norval, M. (1995) The role of *cis*-urocanic acid in UVB-induced immunosuppression. *Adv. in Exp. Med. Biol.* **378**:211.

2. El Ghorr, A. A., Norval, M., Lappin, M. B., and Crosby, J. (1995) The effect of chronic low-dose UVB radiation on Langerhans cells, sunburn cells, urocanic acid isomers, contact hypersensitivity and serum immunoglobulins in mice. *Photochem. Photobiol.* **62**:326.

3. Lappin, M. B., Kimber, I., and Norval, M. (1996) The role of DC in cutaneous immune responses. *Arch. Derm. Res.* **288**:109.

4. Lappin, M.B., Dearman, R. J., Kimber, I., and Norval, M. (1996) Exposure of UVB sensitive mice to immunosuppressive doses of UVB *in vivo* fails to affect the accessory function or the phenotype of draining lymph node dendritic cells. *Expt. Derm.* (in press).

5. Lappin, M. B., Dearman, R. J., Norval, M., and Kimber, I. Strain variations in interleukin-6 production following skin sensitisation of mice. *Immunology* (submitted)

Abstracts:

6. Lappin, M.B., El Ghorr, A. A., Kimber, I., and Norval, M. (1994) The role of *cis*-urocanic acid in UVB-induced immunosuppression. Poster presented at the 3rd International Symposium on Dendritic cells in Fundamental and Clinical Immunology, Annecy, France, 19th-23rd June, 1984.

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8. Lappin, M.B., Dearman, R.J, Norval, M., and Kimber I. (1995) Induced interleukin-6 (IL-6) production in the skin and lymph nodes of UVB resistant and susceptible mice. Immunology **86**: 122 (supplement). Poster presented at the Joint Congress of the British and Netherlands Societies for Immunology, Brighton, UK, 6-8th December 1995.

9. Lappin, M.B., Dearman, R.J., Norval, M., and Kimber, I. (1996) Immunotoxicology of ultraviolet (UV) B irradiation: Correlation with interleukin-6 (IL-6) production. Fund Appl Toxicol **30**: 63-64. Poster presented at the Society of Toxicology Annual Meeting, Anaheim, USA, April, 1196.

10. Norval, M., Lappin, MB., and Kimber, I. (1996) The effect of *in vivo* ultraviolet-B light exposure on accessory function and phenotype of lymph node dendritic cells during the induction phase of contact sensitisation. *J. Invest. Dermatol.* **106**: 921. Poster presented at the Society for Investigative Dermatology, Washington DC, USA, 1st-5th May, 1996.

11. Lappin, M.B., Dearman, R.J., Norval, M., and Kimber, I. (1996) Interleukin-6 production in the skin and lymph nodes of UVB resistant and susceptible mice. *J. Invest. Dermatol.* **106**, 922. Poster presented at the Society for Investigative Dermatology, Washington DC, USA, 1st-5th May, 1996.

THE ROLE OF CIS-UROCANIC ACID IN UVB-INDUCED IMMUNOSUPPRESSION

Michael B. Lappin,[1] Ali El-Ghorr,[1] Ian Kimber,[2] Mary Norval[1]

[1]Edinburgh University, Edinburgh and [2]Zeneca Central Toxicology Laboratory, Macclesfield, U.K.

INTRODUCTION

UV radiation causes many alterations in the skin, including loss of Langerhans' cells (LC) and results also in the accumulation of dendritic cells (DC) in the lymph nodes draining the site of irradiation.[1] One epidermal mediator which may be involved in the induction of UVB effects on LC is cis-urocanic acid (cis-UCA), formed from the naturally occurring trans-isomer following UV-exposure. It has been proposed that UCA acts as a photoreceptor for UV-induced immunosuppression. The action spectrum of UV-induced suppression of contact hypersensitivity is similar to the absorption spectrum of trans-UCA and mice deficient in UCA are resistant to UV-induced immunosuppression. Cis-UCA also has the ability to mimic some of the effects of UV-B such as suppression of delayed-type hypersensitivity responses to herpes simplex virus in mice and depletion of LC from the epidermis.[2] A murine monoclonal antibody, with specificity for cis-UCA has been developed[3] and was used in the present study to elucidate further the role of cis-UCA in modulating immune responses in the skin.

EXPERIMENTAL OVERVIEW

Female C3H-HeN mice received intraperitoneal (i.p.) injections of anti-cis-UCA two hours prior to irradiation with sub-erythemal doses (960 and 1440 J/m^2) of broadband UVB or ear painting with cis-UCA. Control mice were injected i.p. with an isotype matched (mouse IgG1) monoclonal antibody of irrelevant specificity, or with phosphate buffered saline (PBS) alone. Twenty four hours later, LC numbers in the ears were measured by counting ATPase^{+ve} cells in dorsal epidermal sheets (Figure 1). Forty eight hours later, DC numbers in the draining auricular lymph nodes were measured by counting DC microscopically after purification on metrizamide gradients (Figure 2).

RESULTS

Previous evidence has shown that exposure to UVB or ear painting with cis-UCA significantly reduces the density of ATPase^{+ve} LC in the epidermis.[2] In the present study it was found that the depletion of LC induced by either treatment was abrogated by prior i.p.

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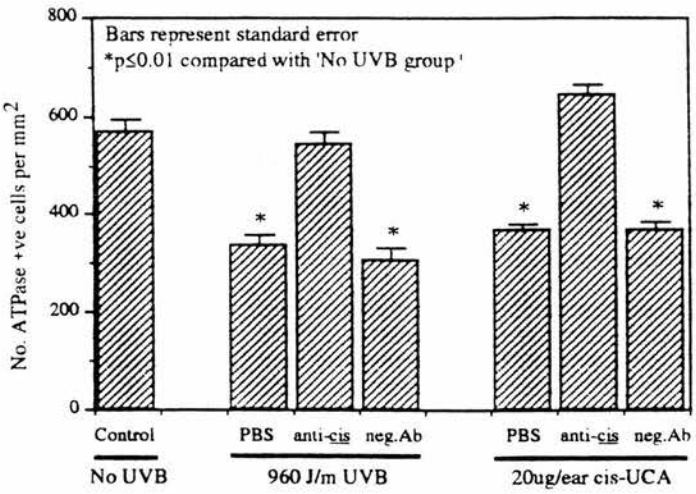

Figure 1. Groups of mice (n=4), received an i.p. injection of PBS, anti-cis-UCA or an irrelevant isotype matched antibody. Two hours later the mice were irradiated with a single dose (960 J/m^2) of broadband UVB or were ear painted with cis-UCA. Control mice received anti-cis UCA but were not irradiated. Twenty four hours later the mice were killed and the ears removed. Dorsal epidermal sheets were prepared and stained for ATPase activity. For each group the number of ATPase^{+ve} cells in 40 fields of view (10 random fields per dorsal epidermal sheet) were counted.

injection of anti-cis-UCA antibody (Figure 1). Injection of an irrelevant isotype matched control antibody or vehicle alone, did not affect the depletion of ATPase^{+ve} cells induced by either treatment. The ability of anti-cis-UCA to block the depletion of ATPase^{+ve} cells in the epidermis was dose dependent (data not shown).

As has been demonstrated previously, exposing the ears to sub-erythemal doses of UVB resulted in a significant increase in numbers of DC found within draining lymph nodes 48 hours later (Figure 2a). Although ear painting with cis-UCA caused a decrease in the number of ATPase^{+ve} cells in the epidermis, it did not induce a subsequent increase in the numbers of DC in the auricular lymph nodes (Figure 2a). In addition, prior injection of the anti-cis-UCA monoclonal antibody did not influence UV-B induced accumulation of DC in the auricular nodes (Figure 2b).

CONCLUSIONS

Following UVB irradiation, cis-UCA is an important mediator in reducing LC numbers in the epidermis. However, cis-UCA is not involved in the UV-induced migration of DC to the draining lymph node. The mechanism by which cis-UCA causes depletion of ATPase^{+ve} cells is unknown but published work has indicated that cis-UCA may act through histamine-like receptors.[2] Other mediators produced in the skin after UVB irradiation may act to induce the migration of DC to the draining lymph nodes. There is evidence that tumour necrosis factor-α(TNFα) production is induced in the epidermis after UVB exposure and that this cytokine may promote migration of DC to the lymph nodes.[4,5] It is possible that TNFα and cis-UCA may modulate immune responses by different mechanisms and the possible interactions between the two mediators are being investigated currently.

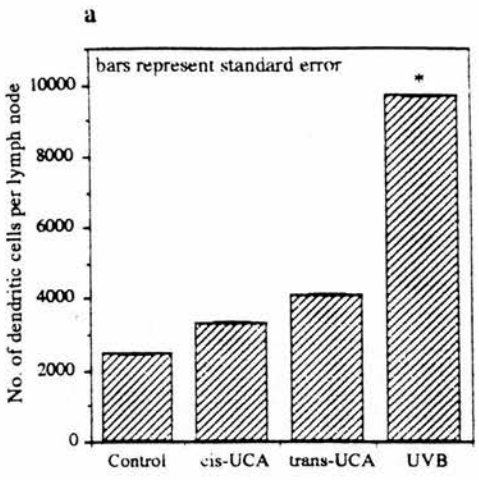

Figure 2a. Groups of mice (n≥6) were painted with 100μg of cis or trans-UCA on both ears or received a single dose (1440 J/m^2) of UVB. Control mice were ear painted with the vehicle alone but were not irradiated.

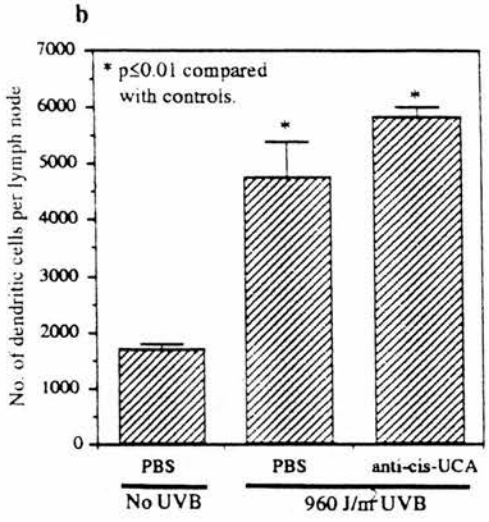

Figure 2b. Groups of mice (n=4) were injected i.p. with PBS or anti-cis-UCA (1:500 in PBS). Two hours later mice received a single dose (960 J/m^2) of UVB. Control mice received anti- cis-UCA but were not irradiated.

Forty eight hours later all mice were killed and the auricular lymph nodes were excised and pooled for each group. Single cell suspensions were prepared by disaggregation through a 70 μm nylon mesh. Dendritic cells were enriched by density gradient centrifugation and counted by direct morphological examination using light microscopy. For each group, five counts were made and the mean number of dendritic cells per lymph node calculated.

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(2)

THE EFFECT OF CHRONIC LOW-DOSE UVB RADIATION ON LANGERHANS CELLS, SUNBURN CELLS, UROCANIC ACID ISOMERS, CONTACT HYPERSENSITIVITY AND SERUM IMMUNOGLOBULINS IN MICE

ALI A. EL-GHORR[1], MARY NORVAL*[1], MICHAEL B. LAPPIN[1] and JOHN C. CROSBY[2]
[1]Department of Medical Microbiology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, UK and
[2]School of Chemistry, University of Bristol, Bristol, UK

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Abstract—C3H mice were irradiated three times a week for up to 6 weeks with either 500 J/m^2 or 1000 J/m^2 broadband UVB (270–350 nm) or 3000 J/m^2 narrowband UVB (311–312 nm; TL01 source). Each dose was suberythemal to the mouse strain used. The number of Langerhans cells (LC) in the epidermis was reduced by over 50% after 2 weeks of irradiation with the UVB source and by 20% following TL01 irradiation. Continued irradiation for up to 6 weeks resulted in no further decrease in LC numbers in the case of the UVB source but a steady decline to 40% in the case of the TL01 source. Sunburn cells were detected following irradiation with both sources but the numbers were very low in comparison with acute exposure. Ultraviolet-B exposure resulted in doubling of the thickness of the epidermis throughout the 6 weeks of irradiation while TL01 exposure did not alter epidermal thickness. Conversion of *trans*- to *cis*-urocanic acid (UCA) was observed with both UVB and TL01 sources. The percentage of *cis*-UCA started to return to normal after 4 weeks of TL01 exposure despite continued irradiation. As observed following a single exposure, the contact hypersensitivity (CH) response was significantly reduced following 6 weeks of UVB irradiation but was unaffected by TL01 exposure, indicating no correlation between *cis*-UCA levels and CH response. Total serum immunoglobulin levels remained unchanged throughout the 6 weeks of UVB or TL01 irradiation but IgE titers significantly increased in all cases in the first 2 weeks of irradiation, indicating a possible shift to a T_{H2} cytokine profile. The IgE levels started to return to normal at later times. Thus chronic broadband UVB exposure induces a number of cutaneous and systemic responses that are likely to be dose dependent, while chronic TL01 exposure induces only some of the these responses.

INTRODUCTION

The effect of UV irradiation on the immune system of mice has been studied mainly using a single exposure, frequently sufficient to induce erythema, or up to four consecutive daily exposures followed by the examination of immunomodulation. Acute broadband ultraviolet-B (UVB)† exposure (270–350 nm) is known to suppress the contact hypersensitivity (CH) response to contact sensitizers[1] and the delayed-type hypersensitivity response to several infectious agents.[2,3] It also reduces the number and function of epidermal Langerhans cells (LC).[4] On UV irradiation, a proportion of the naturally occurring *trans*-isomer of urocanic acid (UCA) found in the stratum corneum is converted to *cis*-UCA.[5] The topical application of *cis*-UCA has been shown to mimic some of the effects of UV irradiation, suggesting that *cis*-UCA acts as one of the mediators of UV-induced immunosuppression.[1,6] In some recent studies, a narrowband UV lamp (TL01) emitting at 311–312 nm has been used rather than a broadband UVB source. Minimal skin injury, as indicated by the induction of edema and sunburn cells (SBC), was found, although efficient conversion of *trans*-UCA to *cis*-UCA was detected.[7] Furthermore, a reduction in the density of LC but not in their antigen presenting function was reported using the TL01 source.[4]

Little evidence is available, however, about the effects of chronic suberythemal UVB exposure over a period of several weeks on the immune system and none about chronic narrowband UVB exposure. High-dose chronic UV exposure is known to lead to premature skin aging.[8] For example, combined chronic UVA (320–400 nm) and UVB irradiation produces a significant increase in the ratio of type III to type I collagen and an increase in fibronectin biosynthesis in the skin of hairless Skh mice, accelerating the modifications observed during aging.[9] Chronic UV exposure can also lead to the development of skin tumors and a contribution of suppressed immunity to the oncogenic process is likely. Thus high-dose chronic UVB exposure of nude mice (15 840 J/m^2 three times a week for 20 weeks) has been reported to induce skin tumors and to suppress basal splenic natural killer cell activity.[10]

Balb/c mice chronically UVB irradiated with an erythemal dose (2250 J/m^2 three times a week) for 1–5 weeks showed

*To whom correspondence should be addressed.
†*Abbreviations:* CH, contact hypersensitivity; Ig, immunoglobulins; IL, interleukin; LC, Langerhans cells; MED, minimal erythemal dose; SBC, sunburn cells; UCA, urocanic acid; UVB, ultraviolet-B radiation.

a significant suppression of their delayed hypersensitivity response to *Mycobacterium bovis*.[11] The antigen presenting activity of lymph node cells from chronically UVB-irradiated C3H/HeN mice (8000 J/m^2 three times a week) was significantly reduced following 4, 12 and 17 weeks of irradiation.[12] A 90% reduction in the number of LC and Thy 1$^+$ epidermal dendritic cells was observed after 1 week of this high-dose irradiation. The LC numbers started to return to normal after 15 weeks of irradiation.[13] Using a lower, but still erythemal, dose of simulated solar UV radiation (2100 J/m^2 UVB and 40 000 J/m^2 UVA five times a week) for 4 weeks reduced the density of LC in both albino and pigmented mice and caused a significant reduction in the CH response.[14]

The present study was performed in order to compare the effects of chronic low-dose broadband UVB and narrowband TL01 exposure with previously published data of acute and high-dose chronic exposure on haired C3H mice. The number of LC and SBC, the thickness of the epidermis and *cis*-UCA conversion during chronic exposure were examined. The CH response following 6 weeks of irradiation and the serum immunoglobulins (Ig) were also investigated in order to assess whether the T_{H1} or T_{H2} arms of the immune response were affected by UV exposure.

MATERIALS AND METHODS

Mice. Male C3H/HeN mice, aged 8–10 weeks, were used throughout. They were bred and maintained in the departmental animal house where they were kept in a 12 h light-dark cycle (shielded fluorescent light tubes) in standard mouse boxes and had unlimited access to food and water. In some experiments, an electrical clipper was used to shave the backs of the mice once a week immediately prior to UV irradiation. Cervical dislocation was performed to kill the mice.

UV irradiation. Mice were exposed to one of two UV sources as already outlined.[4] For broadband UVB exposure, a bank of two TL20W/12 lamps (Philips) was used with an output range of 270–350 nm, peak 305 nm, irradiance 80 μW/cm^2. For narrowband UVB exposure, a single Philips TL01 lamp was employed, which emits predominantly at 311–312 nm, and the total irradiance was 200 μW/cm^2. The output of the sources was determined by Dr. Neil Gibbs (University of Dundee) using a filtered photodiode meter, which was calibrated against measurements made with a UV–visible spectroradiometer (model 742, Optronic Laboratories Inc.) across the spectral range 250–400 nm. Each dose of UVB was either 500 J/m^2 or 1000 J/m^2 and each dose of TL01 was 3000 J/m^2. All UV irradiations were given three times a week (Monday, Wednesday and Friday). One minimal erythemal dose (MED) for this mouse strain was 1500 J/m^2 of UVB and 10 080 J/m^2 of TL01. For comparison, the MED for human type II skin is approximately 900 J/m^2 of UVB and 5000 J/m^2 of TL01. The biological effects of the UVB lamp are therefore approximately six-fold that of the TL01 lamp.[7]

ATPase staining of epidermal sheets. Three days after the last irradiation, the ears of four mice were removed, split and epidermal sheets were prepared from the dorsal side only by floating it epidermal side down in 0.76% tetrasodium ethylenediamine tetraacetic acid for 2 h at 37°C. Epidermal sheets were then carefully removed with a scalpel blade and stained for ATPase using ADP as substrate.[15] The specimens were coded and examined "blind" under the microscope. The number of ATPase$^+$ cells were counted in at least 40 fields per group (1 field = 0.1 mm^2).

SBC density and epidermal thickness. Single ears from each of three mice in each group were removed 3 days after the last irradiation and were fixed in 10% formol saline before routine histological processing. To avoid duplicate sectioning of the same SBC, only every fifth 8 μm section was taken and stained with hematoxylin and eosin. The stained sections were examined on a light microscope attached to a See-Scan Image Analyser. This computer was used to calculate the number of SBC per cm of interfollicular epidermis. Twelve sections were scored for SBC for each group. This represented between 30 and 50 cm of interfollicular epidermis. For each section, the number of SBC/cm was calculated and the data were expressed as mean number of SBC/cm ± SEM. The SBC were characterized as having a pyknotic nucleus surrounded by an eosinophilic and/or vacuolated cytoplasm. In addition the thickness of the epidermis was also measured by the computer at approximately 100 sites per group.

Analysis of UCA isomers. Single ears from each of three mice in each group were collected 3 days after the last irradiation, weighed and their concentration of *cis*- and *trans*-UCA analyzed by HPLC, as already described.[16] The mean amount of each isomer as measured in μg/g wet weight of ear and the percentage present as *cis*-UCA was calculated for each group.

CH assay. In this experiment the backs of the mice were shaved once a week and their ears were not protected from irradiation. The CH response to oxazolone was measured by a standard technique 5 h after the last irradiation as outlined previously.[17] Briefly, nine mice per group received a sensitizing dose of 50 μL 1% oxazolone in acetone/olive oil mixture (4:1) on their shaved backs. A negative control group received 50 μL of the vehicle alone. Six days after the sensitization step, ear thicknesses were measured and all the mice were challenged with 25 μL per ear of 0.25% oxazolone on the dorsal surface. One day after the challenge, all the groups were coded and the ear thicknesses were measured again. The mean increase in ear thickness for each individual mouse was first calculated and then the mean increase for each group of mice.

Serum Ig. Three days after the last irradiation of each group, serum was collected from a minimum of four mice per group and pooled. An ELISA for serum IgE and one for total serum Ig were developed. To measure IgE the plate was coated with 1/500 dilution of the test serum sample in carbonate/bicarbonate buffer overnight at 4°C. Phosphate-buffered saline containing 0.05% Tween 20 was subsequently used as a wash and diluent. Rat anti-mouse IgE (Serotec, UK) at 1/150 dilution was added to the plate and incubated for 5 h at room temperature. After a second wash step, 1/500 anti-rat Ig alkaline phosphatase conjugate (Sigma, UK) was placed in the wells and an incubation carried out for 2 h at room temperature. The substrate was then added after a wash step and allowed to develop. To measure total Ig, a goat anti-mouse Ig (Sigma, UK) was used at 1/1000 to coat the plate overnight at 4°C. After a wash step, the test serum was added at 1/100 dilution and incubated at room temperature for 3 h. Subsequently, a sheep anti-mouse Ig alkaline phosphatase conjugate (Sigma, UK) was added at 1/1000 and incubated at room temperature for 2 h. The substrate was then added. A sheep serum was used as a negative control serum. The mean absorbance of four or six duplicate wells was calculated after subtracting the absorbance of the negative control.

Statistical analysis. The Student's *t*-test was used for analysis of differences in mean responses between groups. Differences were considered statistically significant if $P < 0.05$. The standard error of the means is used in the figures.

RESULTS

The effect of the two UV sources on the number of ATPase$^+$ cells

Only LC in the epidermis express the ATPase enzyme on their cell surface. Ultraviolet-B irradiation with 500 J/m^2 or 1000 J/m^2 three times weekly resulted in approximately 50% and 70% reduction in the density of ATPase$^+$ cells respectively after 2–3 weeks (Fig. 1). This reduction was highly significant and was maintained throughout the UVB irradiation period although there was a tendency for LC numbers to increase as the number of exposures continued. Morphologically, between 20% and 40% of the LC appeared to lose their dendrites following 1–4 weeks of UVB exposure. After 5–6 weeks, however, this number was 7–10%. Exposure to TL01 resulted in a steady decline in the density of LC at the

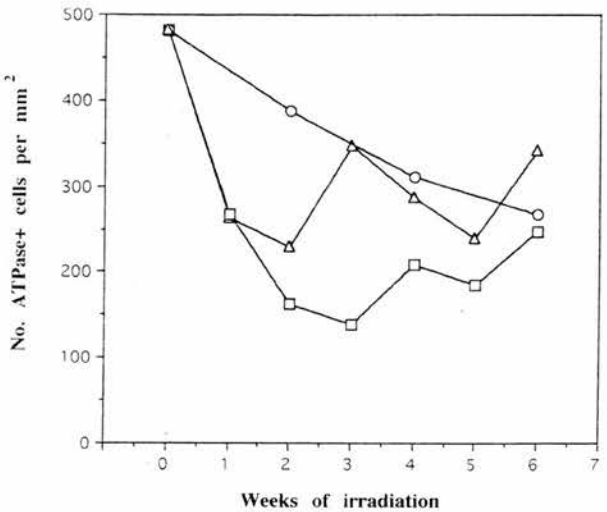

Figure 1. The effect of up to 6 weeks of UV irradiation on the number of LC in murine epidermis. Ears were removed 3 days after the last irradiation and epidermal sheets stained for ATPase enzyme. Triangles represent 500 J/m² UVB, squares 1000 J/m² UVB and circles 3000 J/m² TL01 exposure. The standard error of the mean at each time point was <25. All values were significantly lower ($P < 0.05$) than nonirradiated age-matched controls (time point 0).

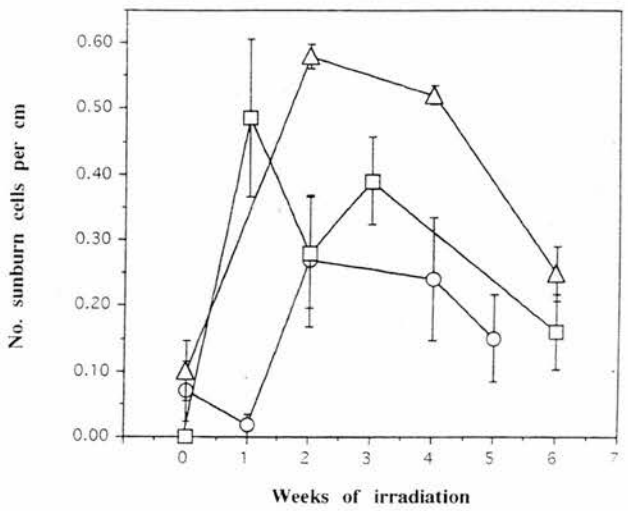

Figure 2. The number of SBC per cm of interfollicular epidermis following irradiation with 500 J/m² UVB (triangles), 1000 J/m² UVB (squares) or 3000 J/m² TL01 (circles). Bars represent the standard error of the mean, n = 12. Three different values at time point 0 are quoted because three control groups were used, one for each irradiation procedure; these three values are not significantly different from each other.

three time points tested (Fig. 1) but there was no effect on the dendritic morphology of LC.

The effect of the two UV sources on SBC and epidermal thickness

Sunburn cells are an indication of the photodamage to the skin following UV irradiation[18] and are thought to represent apoptotic cells. After 1 week of UVB exposure (500 J/m² or 1000 J/m²) SBC started to appear and their numbers increased with further exposure in a dose-dependent manner (Fig. 2). One week of 3000 J/m² of TL01 exposure, however, was not sufficient to induce many SBC. After 2 weeks of TL01, SBC did appear but their numbers remained lower than those obtained following UVB exposure throughout the irradiation period (Fig. 2). After 3 weeks of irradiation, the numbers of SBC started to decline despite continued exposure with all three lamps. The maximum density of SBC obtained after chronic irradiation was 0.58 SBC/cm. In contrast, 24 h after one acute dose of 1500 J/m² UVB, 5.4 SBC/cm were found.

The thickness of the epidermis is an indicator of the response of the skin to UV irradiation. Epidermal thickness increased initially after both doses of broadband UVB irradiation (Fig. 3). The overall increase with 1000 J/m² UVB was significantly higher than with the 500 J/m² exposures and led to doubling of the thickness of the epidermis in these mice. After a slight (but significant) initial increase, TL01 exposure did not markedly affect epidermal thickness (Fig. 3).

The effect of the two UV sources on UCA isomers

Chronic UVB exposure with 500 J/m² or 1000 J/m² increased the *cis*-UCA concentration in mouse ears from 10 μg/g wet weight of tissue to 54–75 μg/g or 160 μg/g, respectively (Fig. 4). This amount of *cis*-UCA was maintained throughout the 6 weeks of irradiation and was equivalent to 25% and 38% of total UCA, respectively. Exposure to TL01 was equally efficient at isomerizing UCA and 170 μg *cis*-UCA per g of ear was detected after 1–3 weeks of irradiation. This was equivalent to 35% of total UCA in this experiment. However, after 5 weeks of TL01 exposure the *cis*-UCA concentration was reduced to 100 μg/g (Fig. 4). The total UCA concentration remained relatively constant throughout the 6 weeks of irradiation.

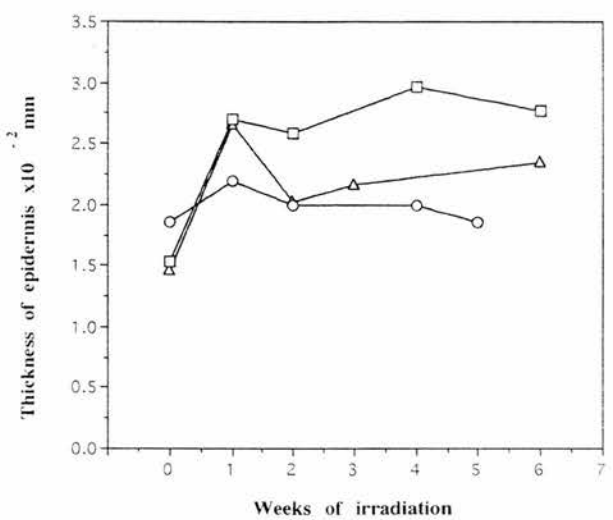

Figure 3. The thickness of the epidermis following irradiation with 500 J/m² UVB (triangles), 1000 J/M² UVB (squares) or 3000 J/m² TL01 (circles) The standard error of the mean at each time point was <0.02. Using the UVB source all values were significantly higher ($P < 0.05$) than nonirradiated controls (time point 0), but only the time point at 1 week was significantly higher using the TL01 source (n = approx. 100).

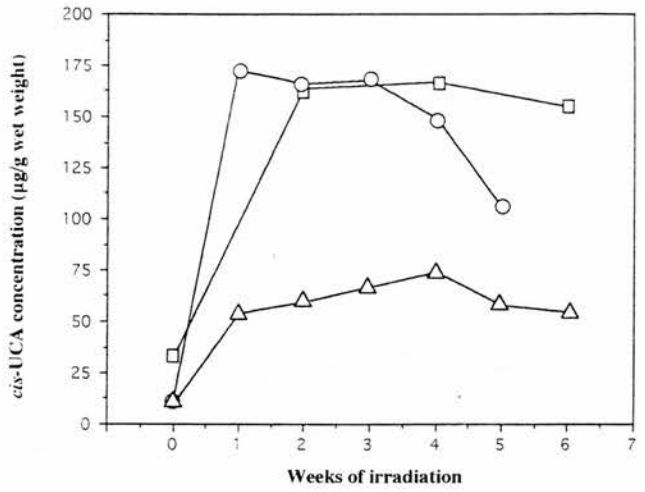

Figure 4. The *cis*-UCA concentration in C3H mouse ears following irradiation with 500 J/m^2 UVB (triangles), 1000 J/m^2 UVB (squares) or 3000 J/m^2 TL01 (circles). The standard errors of all the points on the figure were below 22 μg/g, n = 3.

The effect of the two UV sources on the CH response

Six weeks of chronic UVB exposure with either 500 J/m^2 or 1000 J/m^2 of mice with shaved backs suppressed the CH response to oxazolone by over 60% (Fig. 5). This was statistically significant in both cases. In contrast, 6 weeks of chronic TL01 exposure (3000 J/m^2) had no effect on the CH response to this sensitizer (Fig. 5). Three weeks of chronic TL01 exposure also had no effect on the CH response (data not shown).

The effect of the two UV sources on serum Ig

Both UVB and TL01 chronic irradiation resulted in a substantial increase in serum IgE concentration after 1 or 2 weeks of exposure (Fig. 6a). Thereafter, serum IgE levels gradually returned to normal. Higher titers of serum IgE were detected following irradiation with the TL01 lamp

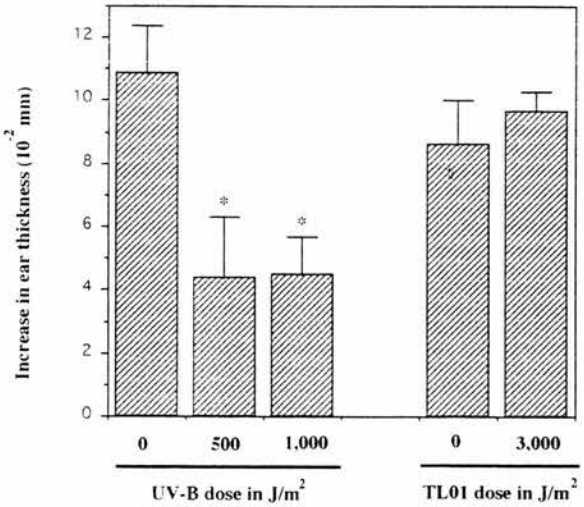

Figure 5. The CH ear swelling response to oxazolone after 6 weeks of irradiation with the UVB and TL01 lamps on shaved dorsal surfaces of mice. The bars indicate the standard error of the mean. *P < 0.05 compared to nonirradiated controls, n = 9.

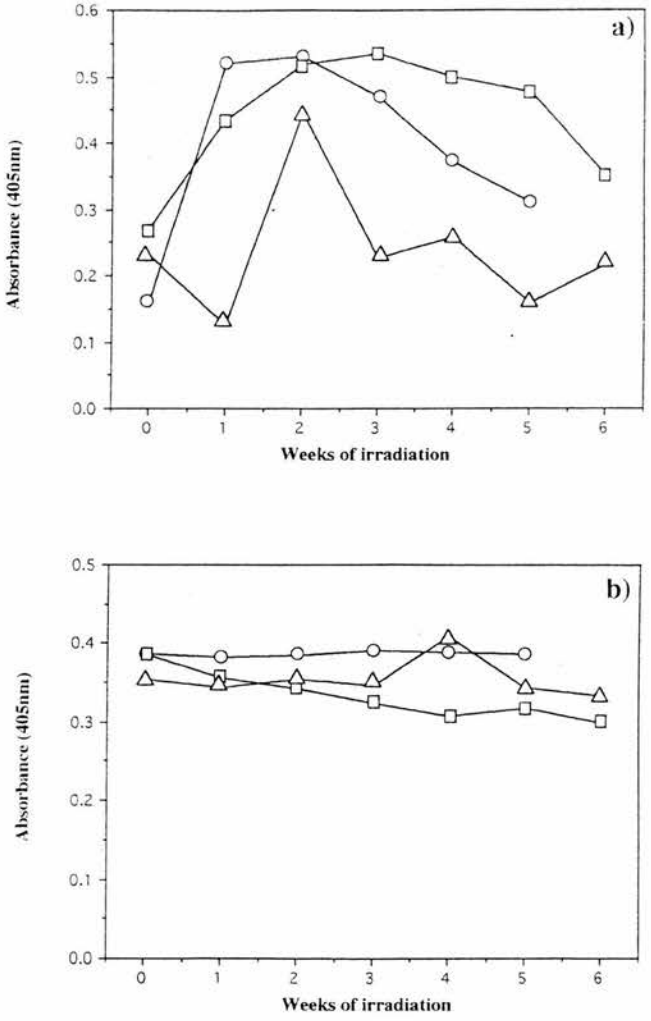

Figure 6. The serum IgE titers (a) and total Ig (b) following irradiation with 500 J/m^2 UVB (triangles), 1000 J/m^2 UVB (squares) or 3000 J/m^2 TL01 (circles). The standard errors of all the points on the figures were below 0.05 absorbance units (n = 6 for IgE and n = 4 for Ig).

(3000 J/m^2) and the 1000 J/m^2 UVB exposures than with the 500 J/m^2 UVB. Total serum Ig, however, remained constant throughout the period of irradiation and with all exposures tested (Fig. 6b).

DISCUSSION

The effect of multiple exposures of mice to broad- and narrowband UVB was investigated. While each single dose of irradiation was suberythemal, it was noted that, after 2 weeks' exposure of shaved mice to doses of 1000 J/m^2 broadband UVB, the skin showed some reddening, before pigmentation developed. The lower dose of broadband UVB (500 J/m^2) did not result in erythema, although tanning was apparent after 3 weeks. Neither tanning nor erythema was seen as a result of TL01 exposure.

As already noted following single doses of broadband UVB or TL01,[4] the density of LC in the epidermis was reduced throughout the irradiation period with both sources. The higher dose of broadband UVB resulted in a greater

reduction in the number of LC. Broadband UVB also affected the dendritic processes of the LC more than TL01. Other workers have shown that erythemal doses of chronic UVB reduce LC density although a return to normal values was noted after 15 weeks of treatment.[13] We did not irradiate animals beyond 6 weeks but after 4 weeks of UVB exposure the numbers of LC started to increase above the minimum levels reached, indicating that LC may have started to repopulate the epidermis. The dendritic morphology of these cells also returned to normal after 6 weeks of UVB irradiation while 40% were rounded at the 4 weeks time point.

The SBC are thought to represent apoptotic keratinocytes, induced in mammalian epidermis in a UV dose-dependent manner.[18] In the present study they were found following broadband UVB and narrowband TL01 exposure, appearing in small numbers when first tested after 1 week, and accumulating thereafter. Irradiation with the TL01 source was slightly less effective than the UVB source at inducing SBC. The number of SBC detected was very low in comparison with acute UVB exposure. There may be a mechanism for removing these dying cells from the epidermis within a few days of their appearance. Alternatively, the cells that give rise to SBC may become more resistant to UV damage after chronic irradiation. In either case, the SBC started to disappear after 3 weeks of irradiation despite continued UV exposure.

Minimal skin damage by the TL01 source is indicated by the epidermis scarcely changing in thickness throughout the study period, while the same was not true of the broadband source. The thickness of the epidermis doubled after 1 week of the 500 or 1000 J/m^2 UVB exposure. This doubled epidermal thickness was maintained with the 1000 J/m^2 dose but was reduced to 1.5 times normal thickness with the 500 J/m^2 dose after the second week. At the time of maximal epidermal thickness, the ears of the mice looked red, as did the backs in the case of shaved animals. Following this, pigmentation developed, which presumably then helped to protect the epidermis from further damage and resulted in the slight reduction in epidermal thickness with the 500 J/m^2 dose of UVB. One study utilizing the TL01 source has reported an increase in epidermal thickness after an accumulated dose of 100 000 J/m^2 is reached.[19] This accumulated dose is equivalent to 10 weeks of irradiation in our experiments and was given to albino hairless Skh mice. For these reasons no direct comparison can be made with the present study. However, in agreement with our results, broadband UVB was shown to be more efficient than the TL01 exposure at inducing thickening of the epidermis.[19] Other studies have examined histological changes in Skh hairless mouse skin following chronic broadband UVB irradiation and have noted, for example, a thickening of the epidermis and an increase in dermal cellularity including inflammatory cells, after 24 weeks of 300 J/m^2 three times weekly.[20] The action spectrum for epidermal thickening in Skh mice has a maximum at 285 nm, with UVB being 50 times more efficient than UVA in this respect.[21] The spectrum for the increase in dermal cellularity has a maximum at 295 nm.[21] Skin thickening is thought to alter the quantity and spectrum of the radiation reaching various layers of the skin, thus making identification of specific chromophores difficult. One such mediator of cutaneous photodamage that has been proposed is non-heme iron, which could act by participating in the formation of reactive oxygen species.[20] Reactive oxygen has been implicated in UV-induced skin damage.[22]

The results from the UCA analysis were somewhat surprising in two respects. First, it has been noted that, in human subjects following insolation[23] and in guinea pigs following irradiation with a hot quartz mercury arc lamp,[24] the total amount of UCA in the epidermis increased several days later. This was presumed to correlate with epidermal thickness. In support of this, the epidermal UCA content of black and Asian skin is reported to be higher than that in Caucasian skin.[25] However, in the present study, we found the total UCA concentration remained unchanged throughout the irradiation period for both UV sources despite an increase in epidermal thickness in the case of the UVB source. Recently, by analyzing the epidermis from different sites of human volunteers we have shown no increase in UCA concentration in areas of the body frequently exposed to sunlight, such as the forehead, compared with areas normally covered, such as the upper thigh (G. Kavanagh, M. Norval, J. Crosby, unpublished). Secondly, while there was an increase in the percentage of *cis*-UCA following 1 week of irradiation with both sources, this proportion did not increase thereafter with further irradiations. Isomerization from *trans*- to *cis*-UCA is known to proceed in a dose-dependent manner until the photostationary state is reached when the ratio of the two isomers is approximately 1:1. A photostationary state was probably not achieved during the chronic irradiation—a maximum of 35% *cis*-UCA was reached with the TL01 and 38% with the UVB (1000 J/m^2). Indeed, in the case of the TL01 source, the concentration of *cis*-UCA began to fall with increasing number of exposures. This cannot be attributed to increased epidermal thickening or tanning since these did not occur with this lamp. We have shown previously that single doses of TL01 and broadband UVB are equally efficient at inducing isomerization of *trans*- to *cis*-UCA.[7] In addition, once the *cis*-UCA is formed, it persists in the epidermis for at least 7 days, returning to background levels at 14 days.[16] It is eliminated mainly by desquamation and in sweat, although there is some evidence that it is present transiently in serum following irradiation.[26] Therefore the dynamics of chronic irradiation with regard to UCA content and percentage *cis*-UCA may be rather different from that found after single exposures. It would be interesting to analyze histidase activity, as well as histidine and histamine concentration in epidermal samples to test changes in metabolic pathways during the irradiation period. Finally the concept of UCA acting as a "natural" sunscreen proposed by Zenisek *et al.*[27] is not supported by our present study because there is no increase in total UCA or *cis*-UCA concentration with time, as might be expected from such a sunscreen.

Since 1983 when *cis*-UCA was first suggested as an epidermal mediator for the immunosuppressive effects of UVB,[1] evidence has been accumulating to substantiate its role in immunomodulation,[9,28] although many other mediators have also been implicated including DNA damage,[29] tumor necrosis factor α,[30] interleukin-10[31] and prostaglandins.[32] In the present study the CH responses to oxazolone were suppressed after 6 weeks of UVB irradiation (500 J/m^2 and 1000 J/m^2) but were not suppressed after 3 or 6 weeks of TL01 irradiation. This result mimics that found

after acute exposure.[4] The ears of the mice were not protected during irradiation, thus exposing the sensitization and elicitation sites to UV light. We have shown previously that this makes no difference to the hypersensitivity response, after a single dose of UV, when the ears are challenged subsequently. In addition, perhaps UV exposure without shielding of particular sites is closer to what happens naturally when animals are solar irradiated, frequently on a daily basis. However, our model does not permit us to distinguish between an effect of UV on the induction or elicitation of the CH response.

As noted above, the TL01 exposure resulted in a loss of LC in the epidermis and therefore, there appears to be no correlation between reduced LC numbers and suppressed CH. The compound *cis*-UCA is induced in the epidermis to approximately the same extent with the two sources and this implies that *cis*-UCA may not be the main mediator that determines the extent of CH responses in the case of chronic irradiation. In support of this, we have shown, from an action spectrum for *trans* to *cis* photoisomerization of UCA in hairless mouse skin, that there is maximal activity at 300–315 nm[33] while the action spectrum for suppression of CH peaks at 260–280 nm.[1] Furthermore, a recent paper indicates that different radiation sources, which all induce approximately 35% *cis*-UCA in the epidermis, varied in their ability to suppress CH with only three sources emitting short wavelengths being effective.[34] Instead a correlation between the severity of edema and degree of CH suppression was found. It is likely that the interaction of *cis*-UCA with cells in the epidermis and elsewhere is complex, and perhaps more steps are required in the process besides the simple presence of *cis*-UCA. For example an appropriate receptor for *cis*-UCA may be of critical importance and irradiation at certain wavelengths may block its action.[34]

Serum IgE concentrations were investigated in the present study as an indicator of T_{H2} activity. Production of the cytokine interleukin (IL)-4 from T_{H2} cells induces activated B cells to switch isotype and produce IgE. Immunoglobulin E is particularly important in hypersensitivity reactions in the skin where it causes mast cell degranulation in the presence of antigen, resulting in histamine release. It has been shown that irradiation of mice with high doses of UVB changes the array of cytokine secretion from a T_{H1} to a T_{H2} pattern with inhibition of IL-2 synthesis and enhancement of IL-4 and IL-6.[35] In addition, low-dose UVB inhibits the capacity of purified LC to induce proliferation of T_{H1} cells while not affecting their ability to stimulate T_{H2} cells.[36] Both sources induced increased serum IgE concentration soon after the UV exposures started. This level returned to normal despite continued exposure. In contrast, total Ig levels in the sera of irradiated mice remained constant, indicating that isotype switching had occurred. It would be worthwhile to analyze the cytokine profile of activated T cells during chronic irradiation to find out whether the IgE results reflect IL-4 production. In conclusion, the two UVB sources used in this study demonstrate different immunomodulatory properties on chronic irradiation, which may indicate the wavelength dependency of some of their effects.

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REVIEW

M. B. Lappin · I. Kimber · M. Norval

The role of dendritic cells in cutaneous immunity

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Abstract This article reviews the role of dendritic cells in cutaneous immunity. Langerhans cells (LC) found in the epidermis are the best-characterized dendritic cell population. They have the ability to process antigen in the periphery, transport it to the draining lymph nodes (DLN) where they are able to cluster with, and activate, antigen-specific naive T cells. During migration LC undergo phenotypic and functional changes which enable them to perform this function. There are other less well-characterized dendritic cells including dendritic epidermal T cells, dermal dendrocytes and dermal 'LC-like' cells. Although there is no evidence that dendritic epidermal T cells (DETC) can present antigen or migrate to lymph nodes, they do influence the intensity of cutaneous immune responses to chemical haptens. Antigen-presenting cells (APC) in the dermis may provide alternative routes of antigen presentation which could be important in the regulation of skin immune responses. Therefore, dendritic cells are vital for the induction of immune responses to antigens encountered via the skin. LC are particularly important in primary immune responses due to their ability to activate naive T cells. The faster kinetics of secondary responses, and the ability of nonprofessional APC to induce effector function in previously activated cells, suggest that antigen presentation in the DLN may be less important in responses to previously encountered antigens. In these seondary responses, dendritic and nondendritic APC in the skin may directly induce effector functions from antigen-specific recirculating cells.

M. B. Lappin · M. Norval (✉)
Department of Medical Microbiology,
The University of Edinburgh, Medical School, Teviot Place,
Edinburgh EH8 9AG, UK
Fax 0131 650 6531

I. Kimber
Zeneca, Central Toxicology Laboratory, Alderley Park,
Macclesfield, Cheshire SK10 4TJ, UK

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Dendritic cells in the skin

The concept that the skin has its own associated immune system was first proposed in 1978 [1]. Langerhans cells (LC) are a constituent part of the skin immune system (SIS), and are found regularly spaced throughout the epidermis, forming a semicontinuous network via long dendritic processes. LC are defined by their dendritic morphology and the presence of a unique intracytoplasmic organelle, the Birbeck granule [2]. In addition to the appearance of LC, they are distinguished by their phenotype, being the only population in normal epidermis to express MHC class II (a molecule involved in the presentation of exogenous antigen to T cells). They originate from bone marrow progenitors, as indicated by their CD45 expression, but are not T-cell derived since they lack the CD3 marker [3]. Human LC express CD1a and stain faintly with anti-CD1c (Table 1) [4]. CD1 molecules show similarities to MHC class I, and may be involved in the presentation of antigen to γ/δ T cells.

Another population of dendritic cells (DC) in the skin is the dendritic epidermal T cell (DETC) which comprise 1–2% of the epidermal cells in rodents [5, 6]. They are bone-marrow-derived ($CD45^+$), belong to the T-cell lineage ($Thy\text{-}1^+$ and $CD3^+$), and express the γ/δ T-cell receptor (TCR) (Table 1). DETC do not express CD4 or CD8 antigens [5], or MHC class II [3]. Their T-cell lineage is confirmed by evidence that the numbers of DETC are significantly reduced in athymic nude mice and that they proliferate in vitro when stimulated with a mixture of concanavalin A and interleukin-2 (IL-2) [5]. γ/δ T-cells are interesting because they recognize a limited set of epitopes including heat shock proteins (HSP), MHC-like molecules and mycobacterial antigens [7]. However, because HSP are produced by both bacterial and mammalian cells, and are highly conserved in both, it has been sug-

Table 1 Phenotype of dendritic cells in the skin (✓ expression, × no expression)

Surface marker	Murine LC	Human LC	DETC	Dermal dendrocyte	Dermal 'LC-like'
MHC class II	✓ [114, 115]	✓ [116]	× [7]	✓ [10]	✓ [12]
MHC class I	✓ [115]				✓ [12]
CD45	✓ [117]	✓ [118]	✓ [5]		✓ [12]
CD3	× [119]		✓ [7]		× [12]
Thy-1	× [119]		✓ [120, 121]		
γδ TCR	× [117]		✓ [122]		
ICAM-1 (CD54)	✓ [79]	✓ [123]		× Induced by IFNγ [124]	
ICAM-2 (CD102)		× [125]			
ICAM-3 (CD50)		✓ [65]			
LFA-1 (CD11a/CD18)	$CD11a^-/CD18^+$ [61]				
MAC-1 (CD11b/CD18)	✓ [119]	✓ [126]		× [124]	$CD11b^-$ [12]
p150/95 (CD11c/CD18)	✓ [127]	✓ [126]			✓ [12]
LFA-3 (CD58)		✓ Weak [128]			
α1 (VLA-1 with β1 chain)		✓ α1β1 weak on 40% LC [129] × [130]			
α2 (VLA-2- with β1 chain)		✓ α2β1 weak on 40% LC [129] × [130]			
α3 (VLA-3 with β1 chain)		✓ α3β1 weak on 40% LC [129] × [130]			
α4 (VLA-4 with β1 chain)	✓ [58]	✓ Weak [128] ✓ α4β1 [129]			
α5 (VLA-5 with β1 chain)		✓ α5β1 [129] × α5β1 [65]			
α6 (VLA-6 with β1 chain)		✓ Weak [128] ✓ α6β1 [129]			
β1		✓ [130]			
CD1a		✓ [4]		✓ [124]	✓ [131]
CD1b		✓ [4]			
CD1c		✓ Weak [4]			✓ [131]
CD44	✓ [58]				
Heat-stable antigen	✓ [132]				
E-cadherin	✓ [55]	✓ [133]			
FcγRI (CD64)					
FcγRII (CD32)	✓ [117]				✓ [131]
FcγRIII (CD16)	✓ [117]				
FcεRI		✓ [134]			
FcεRII (CD23)	× [117]	✓ [134]			
εBP (MAC-2)		✓ [134]			
CD80 (B7-1)	× [93, 94]	× [95]			
CD86 (B7-2)	✓ Weak [93] × [65]				
Factor XIII				✓ [10]	

gested that γ/δ T cells may be involved in autoimmune responses [7]. It has also been proposed that DETC are involved in the induction of tolerance [8], and that the ratio of DETC to LC in the epidermis influences the intensity, but not the duration, of sensitization in mice [9].

A third type of DC present in the skin is the dermal dendrocyte. These cells have been characterized to some extent by their phenotype (Table 1). In human skin they are factor XIII antigen$^+$, sometimes express class II antigen [10] and are $CD1a^+$, $CD1b^+$ and $CD1c^+$ [11]. Factor

XIII is involved in scab formation, crosslinking fibrin with structural proteins, which leads to the conclusion that dermal dendrocytes may be of importance in wound healing. Dermal dendrocytes surrounding the microvasculature in the skin have been called veil cells [10]. Both populations share a common phenotype, but while dermal dendrocytes show both 'dendritic' and 'fibroblast-like' morphology, veil cells are thought to show only 'fibroblast-like' morphology [10]. It has been suggested that dermal dendrocytes are immature precursors of LC. If LC are continuously being replaced by blood-borne bone-marrow-derived DC precursors, then veil cells which are concentrated around the microvasculature, may be DC precursors entering the skin. Besides the dermal dendrocytes, there are other cells in the dermis of mice which express MHC class II, with one subset showing an LC-'like' phenotype (Table 1) [12].

Cutaneous immune responses

Contact hypersensitivity (CH) responses are commonly used as a model to test an animal's or a human's ability to mount a cutaneous immune response. To induce contact sensitivity, the experimental animal is sensitized to a specific hapten by painting a solution containing the chemical onto the skin. Haptens are small molecules which by themselves are nonimmunogenic, but which can act as epitopes when bound to a protein 'carrier'. In the skin haptens acquire immunogenicity by binding to endogenous proteins, which results in the generation of a hapten/protein-specific immune response. Approximately 5 days after the original hapten sensitization, the animal is challenged with a subinflammatory concentration of hapten which within 24 h leads to the generation of a cell-mediated inflammatory response at the site of application. The site of challenge is usually the ears or the foot pad, as it is easy to quantitate the inflammatory reaction by measuring the increase in ear or footpad thickness. Delayed hypersensitivity (DH) responses are broadly similar, but they differ from CH in that the antigen is not a hapten and is administered intradermally. DH inflammatory reactions are characterized by the infiltration of mononuclear cells into the dermis with less cellular infiltration into the epidermis compared with CH responses.

Evidence suggesting that Langerhans cells play an important role in cutaneous immunity

The first clues that LC are involved in cutaneous immunity came from experiments examining contact sensitivity responses in mice. When haptens were applied through sites naturally deficient in LC expression, such as the hamster cheek pouch epithelium and mouse tail skin, specific unresponsiveness to hapten challenge was observed [13, 15]. It was noted that a regime of UVB treatment could artifically deplete LC from normal skin sites [13]. Four consecutive doses of UVB radiation (100 J/m^2 per day) caused a highly significant depletion of ATPase$^+$ epidermal LC in mice [13]. Haptens applied to UVB-treated skin induced hapten-specific unresponsiveness on subsequent exposure at an unirradiated site [13]. The ability of UVB to induce unresponsiveness was dependent on the strain of mouse used, leading to the classification of UVB 'susceptible' and 'resistant' strains. The evidence for this is not as strong as it once was, since resistant strains show significant reduction of CH responses when mice are sensitized with optimal doses of hapten via irradiated skin [16].

These results led to the hypothesis that the suppression of CH is directly related to loss of the epidermal LC. However, there are a number of observations which suggest that other factors are involved also. Firstly, LC are depleted from the epidermis of both susceptible and resistant mouse strains following low-dose UVB treatment [17]. However, resistant mice are able to generate responses to haptens encountered through the depleted skin. Thus, there may be other antigen-presentation pathways available for cutaneous immunity, a concept which is explored more fully later in this review. Secondly, specific tolerance is generated to haptens applied through irradiated skin. This was demonstrated in mice first sensitized to a hapten through skin naturally deficient in LC, or irradiated skin, which showed suppressed immune responses even after they were sensitized again through normal skin prior to challenge [13]. Tolerance generated in this way is associated with the induction of regulatory/suppressor T cells which can transfer suppression of CH [18] and DH responses [19]. Antigen presentation may be taking place but there are likely to be differences in the presentation of antigen encountered via skin depleted of LC (at least in susceptible strains). Thirdly, the immunosuppression generated by UVB is not always confined to skin sites depleted of LC. To examine the local and systemic effects of UVB, mice were exposed to a suppressive dose of UVB, and a contact sensitizer was then painted on to the irradiated site or a distant unirradiated site [20]. When the sensitizer was painted on the skin immediately after the last irradiation, immunosuppression was limited to haptens applied directly to the irradiated site. However, 3 days after the last exposure, there was systemic immunosuppression to haptens applied via unirradiated skin. This evidence suggests that UVB exerts systemic as well as local effects on cutaneous immunity, possibly via soluble mediators.

Antigen processing in the epidermis

The first stage in the recognition of antigen by the immune system is the processing of that antigen by antigen presenting-cells (APC). Processing refers to the internalization and degradation of antigen into immunogenic peptide fragments that can be presented with MHC molecules: it takes place intracellularly in acidified endosomes/lysosomes, although the exact compartment where peptides and MHC class II associate has not been fully defined.

Freshly isolated murine LC are able to process and present the protein ovalbumin (OVA) in its native form, to an OVA peptide-specific MHC-restricted T-cell hybridoma in vitro, an ability which is lost during culture [21]. LC taken from the epidermis and cultured for 72 h are much less efficient at processing native OVA [21]. Similarly, the ability to process antigen from *Leishmania major (L. major)* amastigotes is found in freshly isolated murine epidermal LC, but is lost after 12 h in culture [22]. Haptens such as nickel are able to bind MHC class II-associated peptides directly [23], suggesting that antigen processing may not be required for all forms of contact sensitization.

As LC have phagocytic activity when freshly isolated, it seems likely that epidermal LC have the capacity to process native antigen in vivo. In contrast, like cultured LC, murine lymph node DC are generally poorly phagocytic, although freshly isolated splenic DC show phagocytic activity for zymosan and latex beads [24]. Prior to the work on freshly isolated LC, the lack of phagocytic activity in lymphoid DC led to speculation about the identity of the cell population that processed antigen in the epidermis with the suggestion that keratinocytes may provide this function [25, 26]. Since freshly isolated LC are capable of phagocytosis, it seems unlikely that epidermal LC would require a source of peptides. However, processing by keratinocytes may affect the immune response, depending on whether the peptide fragments are degraded, released into the extracellular matrix or presented on the surface of keratinocytes. Although keratinocytes do not express MHC class II molecules constitutively, they can be induced to do so by interferon-γ (IFNγ) [27]. MHC class II-bearing keratinocytes can provide accessory function for T cells that have been stimulated previously with superantigens or anti-CD3 monoclonal antibody [27]. Keratinocytes are therefore equipped to play an important role in secondary, but not primary, immune responses.

Birbeck granules: markers of antigen processing?

The endocytosis of the CD1 molecule on human LC has been demonstrated using immunogold-labelled anti-CD1 monoclonal antibody and electron microscopy [28, 29]. LC were incubated at various temperatures. At 4°C there was diffuse labelling over the entire cell surface. When the temperature was raised to 15°C, the cell surface labelling was concentrated in clathrin-coated pits and there was internalization of the label in endosomes. At 37°C lysosomes were labelled, and isolated labelled Birbeck granules were visible in the cytoplasm. Another study has examined the endocytosis of Ia antigen by Birbeck granule-like structures in murine DC [30]. Gold-labelled Ia molecules were internalized within Birbeck granules when LC were incubated at 21°C. After 30 min the Birbeck granules disappeared, and the gold label was associated with lysosomes and vacuoles, with aggregates of gold particles on the cell surface. The experiments suggest that Birbeck granules may be important in receptor-mediated endocytosis and intracellular antigen processing. However, there is some evidence that Birbeck granules may not be an absolute requirement for the functional activity of LC. A healthy male subject whose LC lack Birbeck granules has been identified [31]. The LC were present in normal numbers in the skin and expressed CD1a and MHC class II. Functionally, the LC were normal as measured by their ability to induce CH responses in vivo and alloresponses in vitro.

Induction of LC migration

To induce a primary immune response, antigen processed by epidermal cells has to be transported to the local draining lymph node (DLN), where competent antigen-bearing APC can stimulate MHC-restricted proliferation and differentiation of antigen-specific T-cell clones. There is good evidence, that will be presented later in this section, that antigen-bearing LC are able to transport antigen to the DLN. Certainly, LC are capable of migration out of the skin. A number of stimuli cause a loss of LC from the epidermis including exposure to low-dose UVB radiation and skin painting with contact sensitizers [14]. Depletion of murine epidermal LC using these treatments results in a subsequent accumulation of LC and/or DC in the lymph nodes draining the treated site [32, 33], which has been attributed to an influx of DC from the skin. However, skin painting with haptens causes a smaller influx of DC into lymph nodes that drain nonsensitized sites, in addition to inducing an increase in DC numbers in DLNs [34]. This suggests that sensitization may induce systemic LC migration from untreated skin sites. Alternatively, the systemic effects may be due to entry of non-LC-derived DC into lymph nodes.

Keratinocytes provide the necessary microenvironment of the epidermis by producing cytokines which are thought to play an important role in LC migration and differentiation. In response to various stimuli, including UVB radiation and contract sensitizers, keratinocytes express a wide variety of cytokines [35]. Using PCR, it has been shown that topical exposure of mice to contact sensitizers results in increased epidermal mRNA for IL-1α, IL-1β, GM-CSF, TNFα, macrophage inflammatory protein-2 (MIP-2), interferon-induced protein-10 (IP-10) and MHC class II [36, 37]. Tolerogens and chemical irritants also induce an increase in epidermal mRNA for a number of these cytokines [37].

The induction of IL-1β by contact sensitizers is interesting. The earliest change in cytokine mRNA expression in the epidermis is IL-1β, found 15 min after hapten application [37]. This precedes TNFα mRNA expression which is found after 30 min [37]. Depletion of epidermal cell subsets revealed that IL-1β activity is mainly restricted to LC [36]. When IL-1β is injected intradermally, it causes similar changes in cytokine production to those that occur after sensitization, with increased expression of mRNA for IL-1α, MIP-2, IL-10, TNFα and MHC class II, while intradermal injection of IL-1α or TNFα does not affect the cytokine pattern [38]. In addition, a neutralizing

antibody to IL-1β is able to block sensitization [38], suggesting that LC-derived IL-1β is an important initiation signal for the induction of contact sensitization.

The role of TNFα in the migration of LC from the epidermis is controversial. TNFα is induced in human keratinocytes after exposure to UVB radiation [39] and its expression is upregulated by sensitizing chemicals [36]. Intradermal injection of TNFα causes an accumulation of DC in the DLN of mice [40] and a decrease in epidermal LC numbers [41]. Accumulation of DC in the DLN induced by UVB irradiation, contact sensitizers and contact irritants is blocked by pretreatment with neutralizing antibodies to TNFα [42, 43]. Evidence suggests that TNFα may also mediate migration of DC out of the intestine, measured by counting numbers of DC in lymph collected from the thoracic duct of mesenteric-lymphadenectomized rats [44]. Injection of 50 mg endotoxin caused an 8–15 fold increase in DC numbers in the lymph 10–15 h after injection. As with the epidermis, an injection of neutralizing antibody directed against TNFα into these rats prior to the endotoxin abrogated the effects of TNFα. However, in contrast to these results, TNFα has been suggested to act on LC by immobilizing them in the epidermis, as an intradermal injection of TNFα prior to sensitization prevented hapten-induced loss of LC from the epidermis, and so blocked the induction of CH [45].

Other mediators may be involved in reducing the number of LC in the epidermis. One, which may be specific to UVB-induced depletion, is *cis*-urocanic acid (*cis*-UCA). *Trans*-UCA is found in the stratum corneum of the epidermis and is isomerized to *cis*-UCA by UVB irradiation. The *cis*-form is more soluble, being found transiently in the serum of mice after irradiation [46]. *Cis*-UCA mimics some UVB-induced effects on the immune system and causes depletion of LC from the epidermis of mice. The latter effect seems to be specific, as the reduction is abrogated by prior administration of a monoclonal antibody to *cis*-UCA [47]. However, unlike UVB radiation, *cis*-UCA applied topically to the skin of mice does not induce an accumulation of DC in the DLN [33].

The most compelling evidence for epidermal LC migrating to the DLN after antigen challenge, is shown in a model using nu/nu BALB/C mice grafted with skin from C3H mice [48]. When the BALB/c mice were contact sensitized with fluorescein isothiocyanate (FITC) through the graft tissue, the cells binding FITC in the DLN were found to be derived from the C3H graft. Isolated FITC-binding cells from the DLN of BALB/c mice were able to induce a CH response in C3H but not in BALB/c mice. In the same study it was found that at least some of the FITC-binding cells found in the DLN contained Birbeck granules, a feature used to identify epidermal LC. Therefore, it can be concluded that some APC in the DLN are derived from cells in the skin and are LC in origin. However, in another study there were very few donor MHC class II$^+$ cells in lymph nodes draining allogeneic skin grafts [49]. Also, FITC painted on to allogeneic grafts could be found associated with recipient MHC class II$^+$ cells in the DLN, suggesting that some of the FITC reaches the lymph node without binding donor APC in the epidermis [49].

FITC has proved to be a useful hapten in migration studies. It can be painted on to the skin of a mouse and FITC-bearing DC can be visualised in the local DLN. Increasing the dose of FITC increases both the number of DC in the DLN [50] and also the amount of FITC displayed on the surface of the DC [51]. Treatment of mice with monoclonal antibody to MHC class II depletes Ia$^+$ cells in the spleen and lymph nodes but not Ia$^+$ LC in the skin [52]. Skin painting of these mice with FITC resulted in the appearance of Ia$^+$ FITC bearing cells in the lymph nodes that could stimulate an FITC-specific hybridoma in an MHC class II-restricted manner [52].

Although FITC rapidly associates with proteins in vivo, it has been claimed that FITC an move freely to lymph nodes without necessarily binding to LC in the epidermis. After ear painting, FITC found in the DLN is associated predominantly with interdigitating dendritic cells (IDC). Therefore, free FITC entering lymph nodes would have to bind IDC with a high affinity. Although FITC is popular because it can be visualized, other antigens have also been used to study migration. *L. major* amastigotes, administered intradermally, induce migration of DC from the skin to the DLN. DC isolated from these nodes were immunostimulatory for *L. major*-specific T cells [22]. In other studies in sheep and cattle, the afferent lymph veiled cells (ALVC) draining a site challenged intradermally with antigen were collected and used to induce antigen-specific proliferation [53, 54]. ALVC are discussed below where this evidence is reviewed more fully.

Mechanisms of LC migration

Although much is unknown about the mechanism of LC migration, it is likely that adhesion molecules play a role. It has been postulated that the migration signal acts to alter the phenotype of the epidermal LC, causing them to exit from the epidermis. Recent evidence has shown that LC bind to keratinocytes via E-cadherin and that the expression of this molecule is downregulated on LC during culture (Table 2) [55]. Since keratinocytes are the predominant cell population in the epidermis [56], E-cadherin could tether LC in the epidermis and may influence the morphology of LC in the epidermis. Cytokine signals, which could include TNFα and GM-CSF, may induce the downregulation of E-cadherin, weaken adhesive bonds between LC and keratinocytes, and allow migration of LC from the epidermis. ICAM-1, and to a lesser extent LFA-1, may have roles in the migration of LC to local lymph nodes. Intravenous injection of monoclonal antibodies directed against these molecules caused a reduction in the numbers of FITC$^+$ Ia$^+$ DC found in lymph nodes after FITC skin painting, and an inhibition of the induction of CH [57]. Molecules that have been ascribed roles in the homing and recirculation of cell populations, are upregulated on LC during culture. There is increased expression of the surface molecules CD44 and α4 integrin (the

Table 2 Phenotype of various dendritic cell populations in comparison with freshly isolated LC [↑ expression upregulated (compared with LC), ↓ expression downregulated (compared with LC), ✓ expression, × no expression]

Surface marker	Murine DC	Human DC
MHC class II	↑ Lymph node DC [78] ↑ Cultured LC and spleen DC [119] ↑ Spleen DC [61]	↑ Cultured LC [135]
MHC class I	↑ Spleen and thymic DC [115] Spleen DC [61]	↑ Cultured LC [135]
CD45		✓ Blood DC [136] ✓ Blood DC and lymph node DC [118]
ICAM-1	↑ Lymph node DC [79]	✓ Blood and tonsil DC [125]
ICAM-2		✓ Weak blood and tonsil DC [125]
ICAM-3		✓ [65]
LFA-1	✓ Spleen DC [137]	✓ Blood DC [136]
MAC-1	↓ Spleen DC and cultured LC [119]	✓ Weak on blood DC [136]
P150.95	✓ Spleen DC [137] ✓ Cultured LC [127]	✓ Blood DC [136] ✓ 25% of spleen DC [126]
LFA-3		✓ Blood DC [136]
CD1a		× Blood DC [136]
CD1b		✓ Weak on blood DC [136]
CD1c		× Blood DC [136]
CD44	↑ Cultured LC [93]	✓ [65]
CD40	↑ Cultured LC [93]	✓ Cultured LC [138]
Fc Recs	↓ Cultured LC [93]	↓ Cultured LC [138]
CD80 (B7-1)	↑ Cultured LC [93]	↑ Cultured LC [95]
CD86 (B7-2)	↑ Cultured LC [93]	✓ $CD34^+$ progenitors cultured in GM-CSF and TNFα [99]
E-cadherin	↓ Skin-associated lymph node DC [138]	↓ Cultured LC [133]

α-chain of LPAM-1/VLA-4) and ICAM-1 [58]. CD44 is a transmembrane glycoprotein with a molecular weight around 85 kDa [59]. Evidence points to this molecule being involved in cell recirculation by binding specific carbohydrate residues on high endothelial venules [60]. VLA-4 has two ligands, VCAM-1 a cell adhesion molecule induced on endothelium by inflammatory mediators, and the extracellular matrix protein, fibronectin [60]. Adhesive interactions between these molecules on differentiated LC and their ligands may play a role in the migration of LC to the DLN.

Differentiation of LC

The cytokines produced by keratinocytes after treatment with haptens or UVB are important in the differentiation of LC into lymphoid DC. Differentiation of murine LC in vitro requires the presence of GM-CSF, produced by contaminating keratinocytes [61]. Culturing highly purified murine LC in GM-CSF increases their ability to stimulate mixed lymphocyte reactions (MLR) and LC purified from bulk epidermal cell cultures, where contaminating keratinocytes provide cytokines, are also good stimulators of MLR [62]. This ability is not only due to improved viability and increased Ia expression [62]. If LC are cultured in the presence of both GM-CSF and IL-1, a twofold enhancement in their capacity to stimulate MLR is induced compared with LC cultured in GM-CSF alone [62]. Further evidence of a role for GM-CSF in the differentiation/maturation of LC is provided by the inability of LC from unprimed mice to induce an immune response to a tumour-associated antigen, unless the cells are preincubated with GM-CSF [63]. Preincubation of unprimed LC with IL-1α, TNFα, IFNγ and TGFβ does not affect their ability to induce a response and, indeed, some combinations of cytokines cause a reduction in the immune response [63]. If murine LC are cultured in TNFα alone, the viability of the cells is maintained but they do not mature functionally and are poor stimulators of MLR [64]. However, TNF does induce LC differentiation, causing the downregulation of macropinocytosis [65]. Culture of hu-

man DC progenitors, $CD34^+$ cells isolated from peripheral blood, in GM-CSF and TNFα causes their differentiation into cells with DC activity [66].

Other immunomodulatory cell populations in the skin

LC are the principle APC in the skin, but there is evidence that other cells can present antigen and affect cutaneous immune responses. Their contribution to functional antigen presentation may be masked by LC under normal circumstances. However, when the efficiency of antigen presentation by LC is impaired by UVB radiation for example, then the alternative APC may become important.

One cell population of mice and rats, the $Thy\text{-}1^+$ dendritic epidermal T cells (DETC), may induce suppressive immune responses [67] and the ratio of DETC to LC in the epidermis influences sensitization [9]. $Thy\text{-}1^+$ DETC do not express Ia on the cell surface [17] making MHC class II-restricted antigen presentation unlikely, and there is evidence that $Thy\text{-}1^+$ DETC do not migrate to the DLN [41, 68]. Intravenous administration of haptenated $Thy\text{-}1^+$ cells into mice suppresses the induction of CH responses [67, 69]. While in a normal cutaneous response, these suppressor circuits may act to limit an inflammatory cascade, in LC-depleted skin the major signal from the skin may be to suppress the response. It is therefore possible that UVB may be acting via populations other than the LC to induce immunosuppression of cutaneous immunity.

Secondary antigen-presentation pathways have also been proposed as an explanation for the UVB resistance found in certain strains of mice. It has been suggested that UV-resistant mice possess a second antigen-presentation pathway, separate from epidermal LC, possibly mediated by DC in the dermis [70]. Tape-stripped skin which is selectively depleted of epidermal cells, is able to support CH in UVB-resistant, but not UVB-sensitive, mice. However surgical excision of hapten-painted skin within 1 h of application, which removes both the dermis and epidermis, prevents the induction of CH. Recently, it has been shown that dermal cells, haptenated in vitro, from UVB-exposed UV-resistant mice can transfer CH responses to naive mice. In contrast haptenated dermal cells from UVB-exposed UV-susceptible strains are unable to transfer CH and instead induce tolerance [71]. Around 2% of cells in dermal cell suspensions normally express MHC class II; after UVB exposure this drops to around 1.4% in both resistant and susceptible strains [71]. However, the results suggest that in resistant strains the dermal population is able to provide a secondary antigen-presenting pathway, while in susceptible strains these cells induce tolerance. MHC class II^+ LC-like interstitial dendritic antigen-presenting cells have been differentiated from dermal macrophages [12]. Morphology and phenotype were used to distinguish these subsets, with the $CD11b^+$, Ly6c (monocyte/endothelial antigen) subset, thought to be dermal macrophages, having no APC function in syngeneic MLR. The LC-like cells are found in the perivascular and interstitial dermis of resistant and susceptible strains [12].

Other cell populations, not necessarily dendritic, may be involved in presentation of antigen from the skin. One type which appears in the human epidermis during the elicitation phase of the CH response, consists of $CD1^-$ $OKM5^+$ (CD36 monocyte/platelet marker) MHC class II^+ cells [72]. They have been found not to suppress the CH response and have been shown to be responsible for up to 50% of antigen-presentation capacity during hypersensitivity responses [72]. However, a cell population with the same phenotype, which is found in the epidermis after irradiation by certain wavelengths of UV (UVB and UVC) [73], activates a suppressor T-cell population [74]. In another study, UVB induced a loss of cells with LC markers and the appearance of MHC class II^+, $CD1a^-$, $CD36^+$ (on 60% of $CD1a^-$ MHC class II^+ cells), $CD11b^+$ macrophages which may have antigen-presenting activity [75]. Neutrophils (MHC class II^-, $CD11b^+$, $GR\text{-}1^+$), macrophage-like APC (MHC class II^{high}, $CD11b^+$) and macrophages (MHC class II^{low}, $CD11b^-$) infiltrate into the murine epidermis after exposure to UVB [76].

Veiled cells

After an appropriate signal, LC migrate into the afferent lymphatics where they are described as veiled cells (due to their long actively moving processes which resemble veils). In one study the afferent lymph ducts of calves were cannulated, enabling the ALVC to be studied [54]. The ability of the ALVC draining from the site of intradermal antigen challenge [variable surface glycoprotein (VSG) from *Trypanasoma brucei*] to stimulate peripheral blood mononuclear cell (PBMC) proliferation, was measured in monozygotic bovine twins. It was found that they induce proliferation in PBMC from VSG-immunized calves, as rapidly as 30 min after intradermal application of antigens. There was no proliferation of PBMC from VSG-naive calves. Similar work has been carried out in sheep, using the protein antigens OVA and purified protein derivative (PPD) from *bacillus Calmette Guerin* [53]. There was a marked proliferation of OVA- and PPD-specific T-cell lines when incubated with afferent lymph cells from OVA- and PPD-challenged animals. Afferent lymph cells collected prior to challenge did not induce significant proliferation in the antigen-specific T-cell lines. The stimulation was antigen specific as the afferent lymph cells of OVA-challenged sheep did not induce proliferation of PPD-specific T cells, and vice versa. In addition, the ability of ALVC to cluster with primary resting T cells has been described, a property not shared by nonprofessional APC and one of the reasons why antigen presentation by DC is essential for the initiation of primary responses.

The draining lymph node

The veiled cells drain into the paracortical area of lymph nodes where they are called IDC as their dendritic projec-

tions show extensive contact with surrounding cells. The IDC present antigen in a MHC-restricted manner to T cells in the local lymph nodes. T cells which are specific for the antigen/MHC complex and which receive the correct signals from the APC (soluble signals and cell-cell interaction) are induced to proliferative and differentiate.

The changes in the phenotype and function of DC as they migrate to the DLN are thought to be similar to the changes seen in vitro when freshly isolated epidermal LC are cultured. Evidence has been presented in an earlier section showing that LC lose their ability to process native peptide after being cultured for 72 h [21]. However, cultured LC are significantly better at stimulating autologous and allogeneic T-cell responses than are freshly isolated LC. These changes in function are likely to be due to alterations in the phenotype of LC during their migration from the skin and their differentiation into DC. The phenotype of IDC after migration in vivo is similar to the phenotype of LC after differentiation in culture. MHC class II is upregulated on cultured LC [77] and DC isolated from lymph nodes show increased expression compared with epidermal LC [78]. Similarly, ICAM-1 is upregulated on cultured epidermal LC [58] and DC isolated from lymph nodes have higher levels of ICAM-1 than LC isolated from the epidermis (Table 2) [79]. There are also changes in morphology during culture with the majority of LC losing Birbeck granules [80].

Antigen presentation

Antigen presentation is a complex interaction requiring recognition by the T cell receptor (TCR) of peptide antigen bound in the groove of an MHC molecule. Other signals are also required and are provided by soluble cytokines and adhesive associations between the APC and the T cells. During a T-cell/APC interaction, binding of the TCR to the peptide/MHC complex occurs and there are interactions between a variety of adhesion molecules on the T cell and the APC and their ligands [81].

The ability of DC to initiate primary immune responses can be explained, in part, by their capacity to form stable clusters with resting antigen-specific T cells. Splenic DC from mice form clusters with T cells and B cells in vitro in the absence of exogenous antigen [82], while other APC can only cluster with sensitized T cells [83]. Antigen-independent adhesion between T cells and the APC precedes antigen-dependent clustering, and may allow DC to 'sample' different T cells [84]. In vitro assays have shown that clustering occurs prior to, and is essential for, T-cell proliferation [85]. The interaction between LFA-1 and its ligands ICAM-1,2,3 and CD2 and LFA-3 may be important in this first stage of antigen presentation. However, antibodies to LFA-1 fail to block antigen-independent clustering of murine spleen DC although they do block the function of clusters by causing a decrease in cell proliferation and cluster stability [86].

The importance of ICAM-1 expression in antigen presentation has been shown by examining the function of mutant APC from mice which had an 80–95% reduction in ICAM-1 expression [87]. The ICAM-1^{low} APC had a greatly impaired ability to present antigen to T cells. Reconstitution of ICAM-1 by transfection of the gene into these cells restored normal antigen presentation. ICAM-1 induced important costimulatory signals through the LFA-1 molecule on T cells [88]. The ICAM-1/LFA-1 interaction is also necessary in the clustering of T cells with other cells [89], and some groups have suggested that antigen-independent adhesion is the first step toward recognition of the antigen/MHC complex by T cells [81].

There has been much interest recently in the costimulatory functions of members of the B7 family which are ligands for CD28 and CTLA-4 on T cells. B7-2 (CD86) expression is found within 24 h of activation of human B cells, while B7-1 (CD80) expression peaks several days later [90]. B7-2 is found on peripheral blood DC [91], resting human monocytes and on activated T cells, B cells and NK cells [92]. CD28 is expressed widely on both human and mouse resting T cells, while CTLA-4 expression seems to be limited to activated T cells [90]. While both B7-1 and B7-2 are ligands for CD28 and CTLA-4, in vivo interactions may be influenced by the availability of the ligands. B7-2 is found constitutively at low levels on murine epidermal LC, and after 24 h in culture there is a dramatic upregulation in its expression (Table 2) [93]. B7-1 is not found on epidermal LC normally, but it is induced (to lower levels than B7-2) during culture [93–95], and is present on splenic DC [94].

The upregulation of B7-1 and B7-2 during LC culture and their expression on lymphoid DC suggest the involvement of keratinocyte-derived cytokines. B7-2 upregulation can be partially decreased during culture in the presence of an anti-GM-CSF antibody [93], which may reflect a minor role for GM-CSF in the induction of B7-2. Alternatively, the isolated LC may have received a signal to upregulate B7-2 expression during isolation which then may be difficult to reverse [93]. Lipopolysaccharide does not seem to upregulate B7-2 on DC although it does increase the expression of B7-2 on macrophages and B cells [96].

Therefore, both B7-1 and B7-2 are expressed on DC in lymphoid tissue with upregulation during in vitro culture of LC, and a connection with APC activity. Freshly isolated LC are less able to stimulate alloresponses than cultured LC [94, 95, 97] and cultured LC induce alloresponses similar to freshly isolated LC when B7-1 and B7-2 interactions are blocked using CTLA4-Ig [95]. CTLA4-Ig is a fusion protein with the extracellular portion of CTLA-4 spliced to the constant region of the human IgG1 molecule, which acts as a soluble ligand for B7-1 and B7-2 [98]. Interestingly, the alloresponse induced by fresh or cultured LC can be abrogated using CTLA4-Ig and anti-ICAM-1 [95]. The available evidence suggests that CD86 may be the more important costimulatory molecule in alloresponses. The ability of human DC, obtained by culturing $CD34^+$ peripheral blood progenitor cells with GM-CSF and TNFα, to induce alloresponses is inhibited by monoclonal antibodies against CD28 [99]. However,

though monoclonal antibodies against CD80 have little effect on the alloresponse, monoclonal antibodies against CD86 suppress the alloresponse by 70% [99]. When used together, monoclonal antibodies to CD80 and CD86 cause a 90% reduction in alloresponse [99]. CTLA4-Ig binding is completely inhibited in the presence of monoclonals to CD80 and CD86, suggesting that there is not a third ligand for CTLA-4 on DC.

Due to these and other changes in phenotype summarized in Table 2, IDC and cultured LC become specialized at presenting antigen to T cells, and are efficient at activating unprimed lymph node cells and in the induction of alloresponses in vitro [21]. It has been stated that DC are important in the induction of primary immune responses, and good evidence for the role of DC in primary immune responses has been provided using a transgenic mouse model [100]. Transgenic mice were bred that differed in the amounts of I-E MHC expressed on the surface of the major APC populations (DC, B cells and macrophages) [100]. These mice were then immunized with an I-E-restricted peptide antigen. After 8 days the $CD4^+$ T cells were removed from lymph nodes draining the site of immunization and cultured with the immunizing antigen. There was a correlation between the in vitro proliferative response of the $CD4^+$ T cells and the percentage of I-E-expressing cells in the mouse. However, there was no correlation between I-E expression on B cells or macrophages, and the size of the CD4 T-cell recall response.

The fate of the cutaneous DC

DC are not found in the efferent lymph [101] and there are few FITC-bearing DC left in the DLN 6 days after skin painting [102]. It seems likely that some system in the lymph nodes allows the DC to be destroyed without causing damage to the surrounding cells. The clearance of large numbers of inflammatory cells during the resolution of acute inflammation may involve apoptosis of the effector cells followed by ingestion by macrophages [103]. It is possible that DC in the DLN, starved of keratinocyte-derived growth factors which would maintain their viability, undergo apoptosis and are then removed by macrophages without damaging lymph node tissue. Other mechanisms may also act to clear DC from lymph nodes such as activated NK-like cells, or hapten/peptide specific antibody.

Production of a cutaneous immune response

T cells that recognize, and bind to, the specific antigen/MHC complex on the IDC and receive the necessary co-stimulatory signals, are induced to proliferate. Activation signals also induce differentiation of the T cells which undergo changes in both morphology and phenotype. These T cells exit the lymph node in the efferent lymph and enter the bloodstream via the thoracic duct. Recently, activated $CD45RO^+$ T cells, the so called memory subset, have been shown to exhibit specialized recirculation patterns. In the sheep the loss of L-selectin on these cells means that they are less likely to recirculate to peripheral lymph nodes and instead they circulate preferentially through tissue sites including the skin [104].

Commonly, cutaneous immune responses are studied using CH and DH responses as models. The inflammatory response generated during challenge with antigen in both these systems is associated with infiltration of mononuclear cells into the skin. Two subsets of CD4 Th cells have been identified in the mouse: Th1 and Th2 cells [105]. Th1 responses mediate cell-mediated immunity, including CH and DH responses, which are dependent on IFNγ [106, 107]. The cytokines produced by Th2 cells are important in B-cell activation and differentiation and therefore these cells preferentially stimulate humoral immunity. Interestingly, the ability of UVB to suppress sensitization to haptens corresponds to a loss of production of Th1-type cytokines by lymph node cells taken from lymph nodes draining irradiated skin [108]. Although a corresponding increase in Th2-type cytokines has not been detected, it is possible that UVB induces a functional inactivation of Th1-type cells. In support of this it has been shown that the ability of splenic T cells from UV-treated animals to transfer immunosuppression of DH responses to normal recipients, can be blocked by neutralizing antibodies to IL-4 or IL-10 given 4 and 24 h after transfer [109]. Therefore the suppressive action of these T cells on cell-mediated DH responses requires the production of Th2-type cytokines by the T cells.

Secondary cutaneous responses

The antigen-presenting pathway that has been described, LC processing antigen in the periphery and presentation to specific T cells in the DLN, is relevant to all cutaneous immune responses. However, this mechanism may be augmented by alternative antigen-presentation pathways in a secondary response, where there will be a pool of activated or semi-activated T cells specific for the antigen. Earlier, it was noted that subpopulations of these cells show preferential recirculation to tissue sites including the skin. Therefore in a secondary response, T cells could encounter antigen in the periphery bound to LC or other antigen-presenting cell populations such as B cells, macrophages and keratinocytes. Memory/activated T cells expressing CD45RO can be activated by a wide range of APC, while activation of naive T cells requires DC [110]. Therefore skin-homing populations of T cells, after being presented with antigen in the periphery, could release inflammatory factors that initiate a cascade response leading to the migration of effector cells through the endothelium into the skin.

There is some evidence that endothelial cells could present antigen to circulating T cells in the bloodstream. This mechanism would allow for antigen-specific migration of T cells into inflammed tissues. Endothelial cells do not express MHC class II constitutively but this molecule is highly upregulated during infection and allogeneic

transplant rejection [111]. Although cultured cells from virtually any organ can be induced to express MHC class II, endothelial cells are interesting because of the high degree to which class II can be upregulated by cytokines such as IFNγ and IL-1 [112]. As well as adhesive interactions, soluble mediators may be important in antigen presentation. However, cytokines in the blood stream would be rapidly washed away from the inflammatory site. It has been suggested that proteoglycans expressed on endothelial cells might act to bind and present soluble cytokines to circulating leucocytes [113]. Cytokines bind proteoglycans at low-intermediate affinity and some cytokines can specifically bind the glycosaminoglycan sidechains of proteoglycan [113]. Therefore, it is possible that antigen could be presented by endothelial cells in the postcapillary venules to recirculating 'memory' T cells.

Conclusion

The DC of the skin are very important in the production of skin immune responses. The migration of antigen-bearing LC from the epidermis to the DLN and their differentiation into efficient antigen-presenting cells are vital for the induction of primary immune responses. Other antigen-bearing cells, including dermal dendritic cells, may migrate in a similar fashion, and may influence immune responses. The faster kinetics of secondary responses suggest that antigen presentation in the DLN may not be as important as in primary responses. In this case it is likely that DC in the skin, together with other APC, initiate and influence the effector response.

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Exposure of UVB sensitive mice to immunosuppressive doses of UVB in vivo fails to affect the accessory function or the phenotype of draining lymph node dendritic cells

M. B. LAPPIN, I. KIMBER[1], R. J. DEARMAN[1], M. NORVAL.

Department of Medical Microbiology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, Scotland. [1]Zeneca, Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire SK10 4TJ, England.

Correspondence to: Dr. M. Norval, Department of Medical Microbiology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, Scotland. Phone: (0044) 131 650 3167 Fax: (0044) 131 650 6531 E-mail: m.norval@ed.ac.uk

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Abstract

Following the application of sensitizing chemicals to the skin, hapten-bearing Langerhans cells (LC) and possibly other cutaneous dendritic cells (DC) migrate to the draining lymph nodes (DLN) of mice and induce the proliferation of antigen specific effector T cells. This migration of DC to the DLN is required for the induction of primary immune responses. In certain strains of mice irradiation with ultraviolet-B light (UVB) before sensitization results in the suppression of contact hypersensitivity responses. In vitro investigations have suggested that one influence of UVB is to modify the ability of Langerhans cells (LC) to present antigen. In the present investigation putative UVB-induced alterations in lymph node DC in vivo were examined. Lymph node DC were analysed following exposure of C3H/HeN mice to an immunosuppressive dose of UVB (1440J/m^2) 48 and 24 hrs prior to skin painting with the sensitizers fluorescein isothiocyanate or oxazolone. In functional studies, DC prepared from the DLN of contact sensitized mice were examined for their ability to induce hapten-specific secondary T lymphocyte proliferative responses or mixed lymphocyte reactions in vitro. In neither case was the activity of DC influenced by local exposure to an immunosuppressive dose of UVB. The migration of LC from the epidermis to the draining lymph node in response to contact sensitization is associated with increased expression of several membrane determinants necessary for effective antigen presentation, including intercellular adhesion molecule-1 (ICAM-1; CD54), B7-2 (CD86) and Ia antigen. The expression of these molecules was identical on DC isolated from the DLN of UVB irradiated and from control, unirradiated mice. Thus the immunosuppressive effect of UVB on the cutaneous immune system may not necessarily reflect changes in the antigen presenting DC that accumulate in the DLN following skin sensitization.

Keywords: *antigen presentation, contact sensitization., dendritic cells, ultraviolet B irradiation*

Introduction

Cutaneous exposure to contact sensitizers induces the migration of antigen-bearing LC from the epidermis into the afferent lymph and their accumulation as immunostimulatory DC in the DLN (1,2). During migration, LC are subject to changes in both phenotype and function. They become efficient antigen presenting cells and acquire the ability to cluster with, and activate, naive T lymphocytes (3). The ability of DC to induce the expansion of an effector T cell population from naive T cells in the DLN is required for the initiation of primary immune responses. The changes that DC undergo during their migration to the DLN are thougnt to be mirrored by culturing freshly isolated epidermal LC in vitro for several days in the presence of keratinocytes or appropriate epidermal cytokines (4).

Irradiation with ultraviolet B light (UVB) compromises primary cutaneous immune responses in certain strains of mice. These changes correspond to the induction of anergy or antigen-specific unresponsiveness to a variety of antigens, including skin allergens (5). Initially after exposure of susceptible strains to low doses of UVB, the immunosuppression is limited to sensitizers applied locally to the irradiated site (6). It has been known for many years that following UVB irradiation, there is a reduction in the number of LC in the epidermis (7). In addition an increased number of DC accumulate in lymph nodes draining the site of exposure (8). If irradiation is performed prior to skin painting with fluorescein isothiocyanate (FITC) a skin-sensitizing fluorochrome, then a proportion of the increased number of DC found in the DLN bear FITC (8). It seems likely that this hapten-bearing population consists of LC-derived DC, although DC originating from other cutaneous antigen presenting cell populations may be present also.

In vitro studies have shown clearly that UVB is able to inhibit culture-induced expression of a variety of important LC surface molecules, and their activity as antigen-presenting cells (9). As primary immune responses are induced in lymph nodes draining the skin, and lymph node DC are considered essential for the stimulation of naive T lymphocytes, we have questioned in the present study whether irradiation of

C3H/HeN mice with a dose of UVB sufficient to cause the suppression of both contact sensitivity (CH) (10) and delayed-type hypersensitivity responses (11), is associated with changes in the function or phenotype of DC in the DLN. The function of the DC was analysed in two ways. First, the ability of oxazolone and FITC bearing DC from control and irradiated mice to initiate antigen specific secondary proliferative responses by sensitized lymph node cells (LNC) was measured. Second, the ability of DC from unirradiated and irradiated mice to act as allostimulators in a primary mixed lymphocyte reaction was examined. In addition, the phenotypic characteristics of the DC in DLN from irradiated and unirradiated skin were compared. Three determinants were analysed Ia, ICAM-1 and B7-2. Ia was examined due to its role in presentation of antigen to $CD4^+$ T lymphocytes. ICAM-1, is an adhesion molecule known to facilitate the antigen-independent interaction between DC and T lymphocytes (12). Furthermore, expression by LC of this determinant may be important in guiding their directed movement to, and accumulation in, DLN (13), and B7-2 (CD86) provides an important second signal for T cell activation (14).

Methods

Mice

Female C3H/HeN and BALB/c mice, aged 6-8 weeks, were obtained from the specific pathogen-free animal breeding facility at the Medical Microbiology Transgenic Unit, University of Edinburgh.

Contact sensitizers

Fluorescein isothiocyanate (FITC) and 4-ethoxymethylene-2-phenyloxazol-5-one (oxazalone) were obtained from Sigma-Aldrich Co. (Poole, UK). FITC was dissolved in 1:1 acetone:dibutylphthalate at 2.5% w/v and oxazolone was made up in the same vehicle at 1% and 0.25% w/v.

UV Source and exposure

Mice (C3H/HeN strain) were irradiated under two Philips TL-20W/12 bulbs with an output range of 270-350nm, peak 305nm, emitting 80mW/ cm^2. The output of this source was determined using a filtered photodiode meter, calibrated against measurements made with a UV-visible spectroradiometer (model 742, Optronic Laboratories Inc.) across the spectral range 250-400nm. The tube to target distance was 16 cm. One minimal erythemal dose for C3H/HeN mice under these conditions was 1500 J/m^2. To measure the effect of UVB on CH responses the shaved backs of mice were exposed to the 2 doses of 1440 J/m^2 UVB, 48 and 24 hours prior to sensitization. For this experiment the mice were anesthetised by i.p. injection of 0.1 ml sterile distilled water containing 0.83 mg/ml hypnovel (Roche, Welwyn Garden City, UK) and 1.67 mg/ml hypnorm (Janssen Pharmaceutical, Oxford, UK). During the irradiation the mice were contained in a perspex box and their heads were covered with aluminium foil to protect the ears from UVB exposure. Control groups were anesthetised but were not exposed to UVB. In all the other experiments unanesthetised mice were exposed to the same dose of UVB ($2x1440J/m^2$) while being contained in separate compartments of a perspex box to prevent shielding by littermates.

Measurement of CH responses

The backs of C3H/HeN mice were shaved and the mice anesthetised as described previously. One group (n=7) was exposed to the standard dose of UVB (UVB), while the two other groups were unirradiated. Twenty fours later the group exposed to UVB and one of the unirradiated control groups were sensitized on the back with 50µl 1% oxazolone, the backs of the final group were painted with 50µl vehicle. Seven days later the ears of each mouse were measured using an engineers micrometer (Draper SM-510, Japan) and challenged with 25µl 0.25% oxazolone in vehicle. Twenty four hours later the ears of each mouse were remeasured, the increase in ear thickness was calculated, and the results expressed as the mean challenge-induced increase of ear thickness in $mm^{-2} \pm$ standard error.

Enrichment of DC

The method of Macatonia et al (2) was followed in outline. In brief, groups of mice (n=10-15) were exposed to 25µl of the relevant sensitizer, or to an equal volume of vehicle alone, on the dorsum of both ears. In some experiments mice were exposed at the same site to 1440 J/m^2 UVB radiation 48 and 24 hours prior to sensitization. At 18-24 hours after sensitization, mice were killed and their draining auricular lymph nodes excised and pooled for each experimental group. A single cell suspension of LNC was prepared by mechanical disaggregation through 200-mesh stainless steel gauze (J. Stanier and Co. Manchester, UK.). Cells were washed and viable cells counted by exclusion of 0.5% trypan blue. Cells were resuspended in RPMI-1640 (Gibco BRL, Paisley, Scotland) supplemented with 25mM HEPES, 100 i.u./ml penicillin, 200µg/ml streptomycin, 2mM L-glutamine, 100µg/ml gentamicin and 10% heat-inactivated fetal calf serum (RPMI-FCS). DC enriched populations were prepared by density gradient centrifugation on Metrizamide (Nygaard, Oslo, Norway). Briefly LNC were adjusted to $5x10^6$ cells/ml in RPMI-FCS and 8ml of the cell suspension were gently underlayed with 2ml of 14.5% Metrizamide in RPMI-FCS and centrifuged for 20 minutes (600g) at room temperature. The low buoyant density population (DC^+) that accumulated at the interface was collected and washed twice with RPMI-FCS. This population comprised

50-70% DC by morphology using light microscopy. Analysis of these enriched DC populations by flow cytometry revealed a corresponding population of large granular cells with high membrane Ia expression. In some experiments the pellet, which comprised LNC depleted of DC (DC^-), was collected and washed in RPMI-FCS. This population contained around 2% of cells with DC morphology. Flow cytometric analysis confirmed that around 2% of cells in this population had DC characteristics- large granular cells with high Ia expression.

Identification of FITC-bearing cells in the DLN

DC^+ and DC^- cells were prepared as above from the DLN of mice sensitized with 2.5% FITC or 1% oxazolone 18 hours previously. A minimum of 10^4 cells from each population analysed using a Coulter XL flow cytometer. Non-viable cells were gated out, and background controls were set at 1% using DC^+ and DC^- populations isolated from the DLN of oxazolone sensitized mice. DC were identified by forward and side angle light scatter characteristics. The percentage of DC bearing FITC and the intensity of staining were recorded.

Lymphocyte proliferation assay

The method described by Jones *et al* (15) was used. The ears of C3H/HeN mice were painted with 25µl of FITC or oxazolone. Seven days later the mice were killed, and LNC were prepared from the auricular lymph nodes and used as responder populations. They were resuspended at $5x10^6$ cells/ml in RPMI-FCS and 100µl of cells added per well in a round bottomed 96-well plate (Nunclon, Roskilde, Denmark). DC were enriched as described above from C3H/HeN mice sensitized with FITC or oxazolone 18 hours previously. These enriched DC^+ populations were added to wells to provide a DC:responder ratio of either 1:40, 1:55 or 1:100. The effect of UVB was analysed using DC^+ populations from mice given the standard irradiation protocol prior to sensitization compared with DC from mice that received no irradiation. Plates were cultured for 48 hours in a humidified atmosphere of 5% CO_2 in air, and 18 hours prior

to culture termination 0.7μCi ^{3}H-methyl thymidine (Amersham Life Science, Little Chalfont, UK) was added to all wells. The cells were harvested using an automatic cell harvester (Dynatech) and ^{3}H-methyl thymidine incorporation measured by β-scintillation counting. Results were expressed as the mean cpm± standard deviation (s.d.) from 5 replicate cultures.

Mixed lymphocyte reaction

Responder LNC were prepared from pooled auricular lymph nodes isolated from BALB/c strain mice (n=3-5) which had been painted 48 hours earlier with 1% oxazolone on the dorsum of both ears. LNC were prepared as described above and cultured at $1.5x10^5$ cells/ml in RPMI/FCS. DC^+ populations were prepared from the auricular lymph nodes of unsensitized C3H/HeN mice (n=10-15) exposed to low dose (1440 J/m^2, -48, -24 hours) or high dose (15 kJ/m^2, -24 hours) UVB treatment. Control DC^+ populations were enriched from the skin draining lymph nodes of unirradiated and unsensitized mice (n=10). Cells with DC morphology were counted and a known number of C3H/HeN DC were cultured with BALB/c responder LNC at various stimulator: responder ratios. DC^- populations were also used as stimulators. These cells were cultured alone or with BALB/c LNC at matching stimulator:responder ratios as for the DC^+. Plates were cultured for 120 hours in a humidified atmosphere of 5% $C0_2$ in air. ^{3}H-methyl thymidine (Amersham Life Science) was added to all wells for the last 24 hours of culture. The cells were then harvested and the thymidine incorporation was measured as described above.

Phenotypic analyses

DC were enriched, as described above, from the auricular lymph nodes of C3H/HeN mice 18-24 hours after sensitization with oxazolone (n=10-15). In some experiments mice were exposed to UVB 48 and 24 hours before sensitization. A minimum of 10^5 DC were incubated on ice for 30 minutes with the appropriate isotype control, rat IgG2a specific for human MHC class I or rat IgG2b raised against dinitrophenyl (Serotec,

Oxford, UK), or with monoclonal antibodies recognising Ia, B7-2 or ICAM-1. Rat-anti-mouse monoclonal antibodies recognising Ia (Serotec, Oxford, UK) and B7-2 (CD86) (Pharmingen, San Diego, USA) both IgG2a isotype were purchased, and the ICAM-1 monoclonal, IgG2b isotype, was acquired as a hybridoma culture supernatant (16). The cells were washed once with 2ml of RPMI-FCS and incubated with an affinity purified $F(ab')_2$ rabbit anti rat IgG-FITC conjugate (Serotec, Oxford, UK) for 30 minutes on ice. Finally, the cells were washed once more in RPMI-FCS, fixed in 1% formyl saline and analysed using a Coulter XL flow cytometer. Non-viable cells were gated out, and isotype controls were routinely set at 1%. DC were identified by forward and side angle light scatter characteristics. The percentage of DC expressing each marker and the density of expression (mean fluorescence intensity) of the marker were recorded. A minimum of 5000 events were accumulated using logarithmic amplification of fluorescence intensity.

Statistics

P values were calculated using an unpaired Students t-test in Statworks 1.1.

Results

Effect of UVB on contact hypersensitivity responses in C3H/HeN mice

As has been shown previously (10), exposure of mice to 1440 J/m^2 UVB on two successive days prior to sensitization with oxazolone resulted in a reduction in contact hypersensitivity measured as a function of challenge-induced increases in ear thickness from 11.14±1.34 to 5.83±1.25 mm^{-2} 24 hours after oxazolone challenge. This represents a 47% suppression of hapten-specific ear swelling (p=0.01).

Identification of hapten-bearing DC in the DLN of sensitized mice

To determine whether the accumulation of DC in the DLN after sensitization could be attributed to an influx of DC from the skin, mice were painted on the dorsum of both ears with FITC or oxazolone; 18 hours later draining auricular lymph nodes were removed and DC$^-$ and DC$^+$ populations were prepared. Background fluorescence levels were set at 1% on DC$^-$ and DC$^+$ populations from oxazolone sensitized mice. Around 28% of LNC in the DC$^-$ population had FITC on their surface but the intensity of staining was low with a mean fluorescence of 1.12 on a $\log_{10}$ scale (data not shown). Around 37% of all the cells in the DC enriched population had FITC on their surface and the intensity of staining was high with a mean fluorescence of 24.7 on a $\log_{10}$ scale (Fig 1). In addition, 73% of cells in this population with DC size and granularity characteristics bore FITC (data not shown).

Effect of UVB on the ability of DC to stimulate in vitro secondary lymphocyte proliferative responses

Initial experiments to optimise the system resulted in the use of responder cells at 5x10^5 cells/well and enriched DC at 1:55 stimulator to responder ratio, which produced a response of around 15000 CPM (data not shown). When DC$^-$ populations were used as stimulator cells, they failed to induce proliferative responses over background.

Fig 2 shows the results of two independent experiments in which enriched FITC-DC isolated from control and UVB irradiated mice were used to stimulate the proliferation of LNC from FITC-sensitized mice. In the first experiment (Fig 2a) there was no significant difference between the accessory cell activity of DC isolated from control or irradiated mice. In the second experiment (Fig 2b) there was a statistically significant difference ($p=0.03$) between the proliferative responses induced by DC from control and irradiated mice. However, differences were very modest, a 16% reduction in proliferation when DC from irradiated mice were used as stimulator cells compared with DC from control mice. In two further experiments, oxazolone bearing DC from UVB irradiated mice induced equivalent antigen-specific proliferative responses to oxazolone-DC from unirradiated mice (data not shown). These experiments were identical to those shown in Fig 2, except that the responder cells were used at a concentration of 2.5×10^5 cells/well.

Since the assays shown in Fig 2 were conducted using concentrations of DC that provoked good proliferative responses, it was decided to determine the effect of UVB on proliferation when DC numbers were limiting. In fig 3, DC were used at 2 stimulator:responder ratios, 1:100 and 1:55, and the antigen specificity of the response was examined by using oxazolone and FITC-bearing DC as stimulator cells for LNC from oxazolone-sensitized mice. Figure 3a shows the proliferative response of LNC cultured in the presence of oxazolone-bearing DC from control and irradiated mice. Oxazolone-bearing DC stimulated good proliferative responses by LNC from mice sensitized with oxazolone in a dose-dependent manner. UVB treatment did not inhibit this ability. The failure of UVB to affect the accessory activity of DC was seen when DC were present at both stimulator:responder ratios. The hapten specificity of the response is shown in Fig 3b. Although FITC bearing DC were able to induce limited antigen non-specific proliferative responses from oxazolone sensitized LNC, their capacity to induce proliferation was poor when compared with the antigen specific proliferative responses illustrated in Fig 3a.

Effect of UVB on the stimulatory capacity of DC in antigen primed primary mixed lymphocyte reaction

When enriched DC from C3H mice were cultured with LNC from BALB/c mice sensitized previously with oxazolone, significant proliferative responses were seen using a range of stimulator to responder cell concentrations (1:15 to 1:100) (Fig 4). The stimulation of alloresponses was dependent upon the presence of DC, as stimulator populations depleted of DC failed to induce proliferative responses (Fig 4). However, when DC were isolated from mice given a suppressive dose of UVB radiation, their ability to induce alloresponses was unimpaired. Even exposing mice to much higher doses of UVB 15 kJ/m^2 24 hours prior to enrichment of DC from the draining lymph nodes failed to effect the function of the DC as allostimulator cells (Fig 5).

Ia, ICAM-1 and B7-2 expression on DC enriched from the lymph nodes of unirradiated, sensitized and UVB irradiated sensitized mice

The migration of LC from the skin and their accumulation as immunostimulatory DC in the draining lymph nodes is accompanied by the increased expression of several membrane determinants considered necessary for the effective induction of T lymphocyte responses including Ia, ICAM-1 and B7-2. Exposure of mice to UVB under conditions that result in immunosuppression of CH failed to influence the expression by lymph node DC of any of these determinants (Fig 6). For each of the three determinants tested, UVB treatment did not affect the % of DC expressing the determinant, or the density of expression.

Discussion

UVB exposure causes the suppression of cutaneous immune responses to a variety of agents. It has been hypothesised that UVB exposure affects antigen-presenting cell populations in the skin, causing them to induce inappropriate effector responses following their migration to DLN. In order to investigate whether altered DC populations could be mediators of UVB-induced immunosuppression, the phenotype and function of DC populations in the DLN were examined after exposure to UVB light in vivo.

Although the presence of FITC-bearing DC in the DLN of FITC-sensitized mice is not necessarily a marker for epidermal derivation, it is likely, though still a matter of some contention, that the majority of these DC cells bind FITC in the skin prior to their migration to the DLN. Macatonia *et al* (17) reported two populations of $FITC^+$ DC in the DLN after skin painting, one with low levels of FITC and another intensely fluorescent population. The DC bearing high levels of FITC first appeared 2-8 hrs after skin painting and induced T cell proliferation, the other population appeared 30 minutes after skin painting but were unable to induce proliferative responses. Kripke *et al* (1) reported two populations of $FITC^+$ DC in the DLN after skin painting; 75% of these DC expressed the antigen F4/80 a marker of LC but not lymph node DC which suggests they are a skin derived population. The remaining 25% of the DC did not express F4/80 and may represent a resident lymph node population. These and other studies suggest that the majority of DC in the DLN after skin painting with a contact sensitizer are skin derived, and therefore it was considered that they constitute an appropriate antigen presenting cell population with which to examine the immunomodulatory effects of UVB in vivo.

Using an immunosuppressive dose of UVB, the ability of FITC or oxazolone-bearing DC to initiate antigen-specific proliferation in vitro was examined. Initial results confirmed the ability of both FITC and oxazolone-bearing DC to initiate antigen-specific proliferation of LNC from contact sensitized mice, and in accordance with previous results, FITC and oxazolone-bearing DC showed a limited antigen non-specific

stimulatory capacity (data not shown for oxazolone-bearing DC) (15). However, UVB failed to influence materially the ability of DC to initiate antigen specific proliferative responses. This contradicts the results of Bucana *et al* (18) where exposure of mice to 400 J/m^2 UVB on 4 consecutive days prior to FITC sensitization reduced the ability of enriched DC to induce proliferative responses in FITC specific T cell lines. After metrizamide gradient enrichment, the cell population contained 10-30% DC and was used at a ratio of stimulator: responder cells of 5:1. In our study, the enriched population contained 50-70% DC on basis of morphology and was used at a ratio of 1:55 or 1:100. It is possible that the suppression of proliferative responses reported by Bucana *et al* could be a result of UVB-induced changes in the large number of non-DC contaminating the stimulator cell population in the assay. Alternatively the responding cell population, which was FITC-specific T cell lines in the Bucana *et al* study and LNC stimulated in vivo with FITC in our study, could interact differently with DC through, perhaps, the differential expression of various co-stimulatory molecules.

The accessory cell function of DC in a primary allogeneic immune response was examined also. It was found that LNC prepared from BALB/c mice sensitized with oxazolone could be stimulated to proliferate in an antigen non-specific manner using lymph node DC enriched from unsensitized C3H/HeN mice. It was shown that DC isolated from mice that had received a suppressive dose of UVB displayed no loss in their ability to act as accessory cells.

In addition, the expression of three markers (MHC class II, ICAM-1 and B7-2) which are important in the physical interaction and signalling between antigen presenting cells and T lymphocytes was examined. No changes in the percentage of DC expressing these membrane determinants or the density of expression were found after exposure to UVB.

A number of studies have documented the effects of in vitro UVB exposure on epidermal LC. Irradiation of human epidermal cell suspensions (8-20% LC) and purified LC populations (70-90% LC) with 100-200J/m^2 of UVB in vitro significantly inhibits their ability to stimulate primary alloresponses, mitogen-induced proliferation

and proliferative responses to recall antigens (19). Similarly, exposure of murine epidermal cell suspensions to a low dose of UVB (25 J/m^2) inhibits anti-CD3 induced proliferation of T cells, while 100-200 J/m^2 abrogates completely their stimulatory capacity (9). It has also been demonstrated in vitro that UVB (50-100 J/m^2) inhibits the up-regulation of intercellular adhesion molecule-1 (ICAM-1) expression by LC that is normally induced during culture (9) or during the migration of LC to the DLN following skin sensitization (20). However, in vitro exposure of LC to 100 J/m^2 of UVB is cytotoxic to LC, causing their eventual loss from cultures after 48-72 hours (21).

It is believed that many of the DC found within the skin-draining lymph nodes derive from epidermal LC. Therefore it is interesting that the susceptibility of LC to UVB in vitro is not mirrored by similar changes in lymph node DC following exposure to UVB in vivo. There may be a number of explanations for this observation. In the in vitro studies, LC in suspension were exposed directly to UVB at doses that are cytotoxic. Therefore, the results obtained in vitro may reflect responses to cellular injury. When animals are irradiated, UVB loses much of its energy as it penetrates through the layers of stratum corneum. Therefore, in addition to the direct damage to DNA that UVB can cause, UVB induced mediators may be important, especially in the immunosuppression following low doses of UVB. Secondly, LC that are directly damaged by UVB may be unable to migrate to DLN. Low doses of UVB inhibit the migration of cells out of human skin explants in vitro, and the proportion of the migrating cells expressing the LC marker CD1a are slightly reduced (22). Finally, it has been proposed that many of the DC which accumulate in DLN are of dermal rather than epidermal origin, though this has not been shown directly.

It is not possible therefore on the basis of immunostimulatory activity and expression of Ia, ICAM-1 or B7-2 to reconcile the immunosuppressive properties of UVB in vivo on the basis of alterations in DC phenotype or function. However, the measurements performed in this study could have failed to detect more subtle changes in DC induced by UVB. There is evidence that local exposure to UVB causes the preferential

production of Th2 type cytokines by DLN cells (23,24). Irradiation may alter the DC arriving in the DLN, or alternatively change the lymph node microenvironment, causing the selective activation of a particular T cell subset. Therefore, measuring the ability of DC to stimulate proliferative responses may be of less importance than examining the functional activity of the effector T cells induced to proliferate by the DC. In addition, although UVB did not alter the expression of the three surface molecules examined here, it cannot be concluded that UVB will not influence other surface molecules, such as B7-1 (CD80) and heat stable antigen both of which have costimulatory function (25). Finally, by testing the function of DC in vitro, the effect of the lymph node microenvironment is ignored and the results may not reflect the antigen-presenting activity of DC in vivo.

These data reveal that the marked changes in LC phenotype and function induced by in vitro UVB exposure are not apparent in DC populations in lymph nodes draining the site of exposure to UVB in vivo. In vitro UVB results in a substantial decrease in LC accessory cell activity and a reduction in the culture induced up-regulation of certain surface molecules. If these UVB-induced changes which are seen in vitro were mirrored by DC migrating to the DLN after irradiation in vivo, it would be possible to speculate that these DC would fail to induce appropriate effector T cell responses. However, this seems not to be the case since DC which accumulate in the DLN of C3H/HeN mice following irradiation in vivo are identical in function and phenotype to DC from control mice. Caution must be exercised therefore when using in vitro data to investigate the complex responses seen in vivo after exposure to UVB.

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Figure 1. The expression of FITC on DC enriched from the DLN following skin painting with FITC. Mice were ear painted with oxazolone or FITC. 18 hours later enriched oxazolone (filled area) and FITC (open area) bearing DC populations were prepared from lymph nodes and their fluorescence was analysed using a flow cytometer.

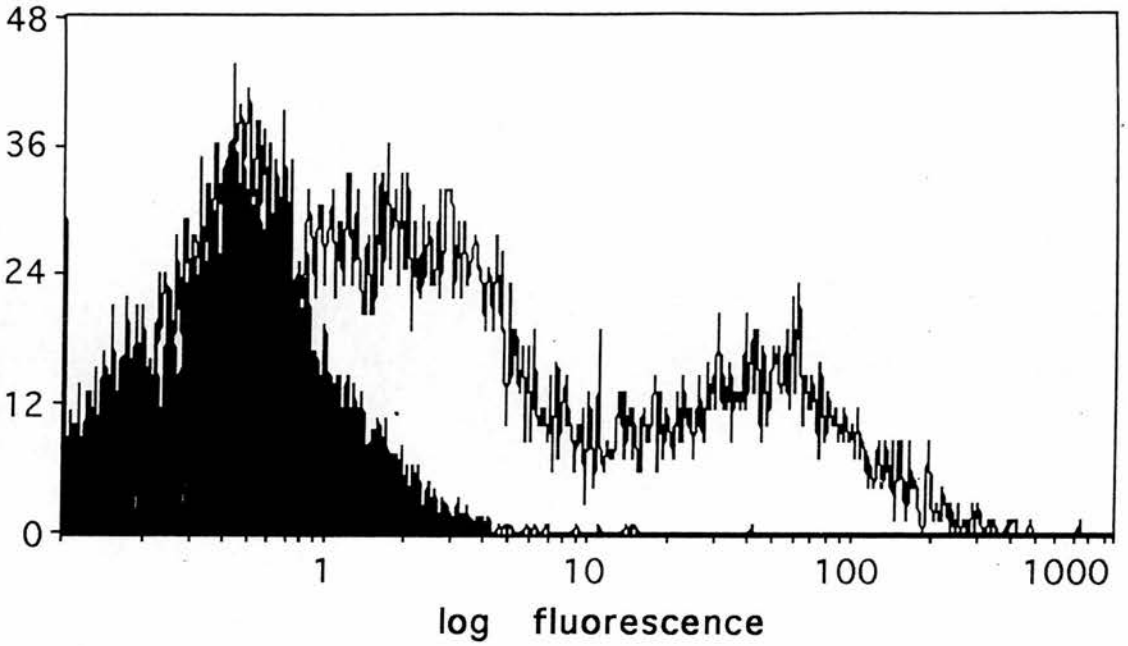
Figure 2. The effect of UVB on the ability of DC enriched from mice sensitized with FITC to stimulate FITC specific lymphoproliferation in vitro. The results from two separate experiments are shown in Figure 2a and 2b. FITC sensitized responder LNC were cultured alone (□), with FITC bearing DC from unirradiated mice (▨), or FITC bearing DC from mice exposed to UVB (2880 J/m^2 total) prior to skin painting (▩). The cells were cultured for 48 hours and ^{3}H-methyl thymidine was added to all wells 18 hours prior to termination of culture. The results are expressed as mean CPM±SD for 5 replicate cultures. The proliferative CPM for DC cultured alone were below 500.

Figure 3. The effect of UVB on the ability of DC enriched from mice sensitized with oxazolone or FITC to stimulate oxazolone specific lymphoproliferation in vitro. In Figure 3a, oxazolone sensitized responder LNC were cultured alone (□), with oxazolone bearing DC from unirradiated mice (▨), or oxazolone bearing DC from mice exposed to UVB (2880 J/m^2 total) prior to skin painting (▩). Figure 3b is the same except that FITC bearing DC from unirradiated mice (▨), or FITC bearing DC from mice exposed to UVB prior to skin painting (▩) were used instead of oxazolone bearing DC. The cells were cultured for 48 hours and ^{3}H-methyl thymidine was added to all wells 18 hours prior to termination of culture. The results are expressed as mean CPM±SD for 5 replicate cultures. The proliferative CPM for DC cultured alone were below 500.

Figure 4. The effect of low dose UVB on the allostimulatory capacity of enriched DC in vitro. Responder LNC from oxazolone sensitized BALB/c mice were cultured alone or with various numbers of DC enriched (DC^+) from unirradiated C3H mice (■) or UVB (2880 J/m^2 total) irradiated C3H mice (▲). Responder LNC were also cultured with DC depleted (DC^-) populations from unirradiated (□) and UVB irradiated mice (△). The cells were cultured for 120 hours and ^{3}H-methyl thymidine was added to all wells 24 hours prior to termination of the culture. The results are expressed as mean CPM±SD for 5 replicate cultures. The proliferative CPM for DC^+ and DC^- cells cultured alone were below 500 CPM.

Figure 5. The effect of high dose UVB on the allostimulatory capacity of enriched DC in vitro. Responder LNC from oxazolone sensitized BALB/c mice were cultured alone or with various numbers of DC enriched (DC^+) from unirradiated C3H mice (■) or UVB (15 kJ/m^2 total) irradiated C3H mice (▲). Responder LNC were also cultured with DC depleted (DC^-) populations from unirradiated (□) and UVB irradiated mice (△). The cells were cultured for 120 hours and ^3H-methyl thymidine was added to all wells 24 hours prior to termination of the culture. The results are expressed as mean CPM±SD for 5 replicate cultures. The proliferative CPM for DC^+ and DC^- cells cultured alone were below 500.

Figure 6. The effect of local UVB exposure on the phenotype of DC enriched from the draining lymph node. Mice were unirradiated or exposed to UVB (2880 J/m^2 total) prior to ear painting with oxazolone. DC were enriched from draining lymph nodes 18-24 hours later. The phenotype of the DC was analysed using flow cytometric analysis. Ia (a), ICAM-1 (b) and B7-2 (c) expression on DC from control (thick solid line) and UVB irradiated mice (thin solid line) was examined. The appropriate isotype controls were included for control DC (thick hatched line) and UVB DC (dotted line). One representative experiment is shown, the results were reproduced on three separate occasions.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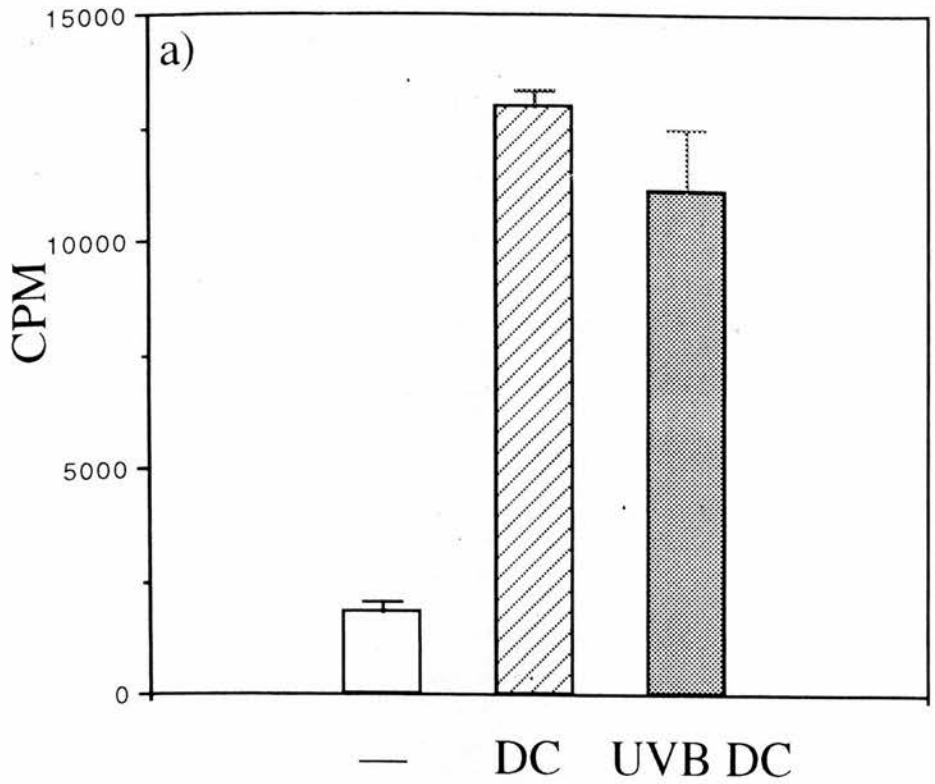
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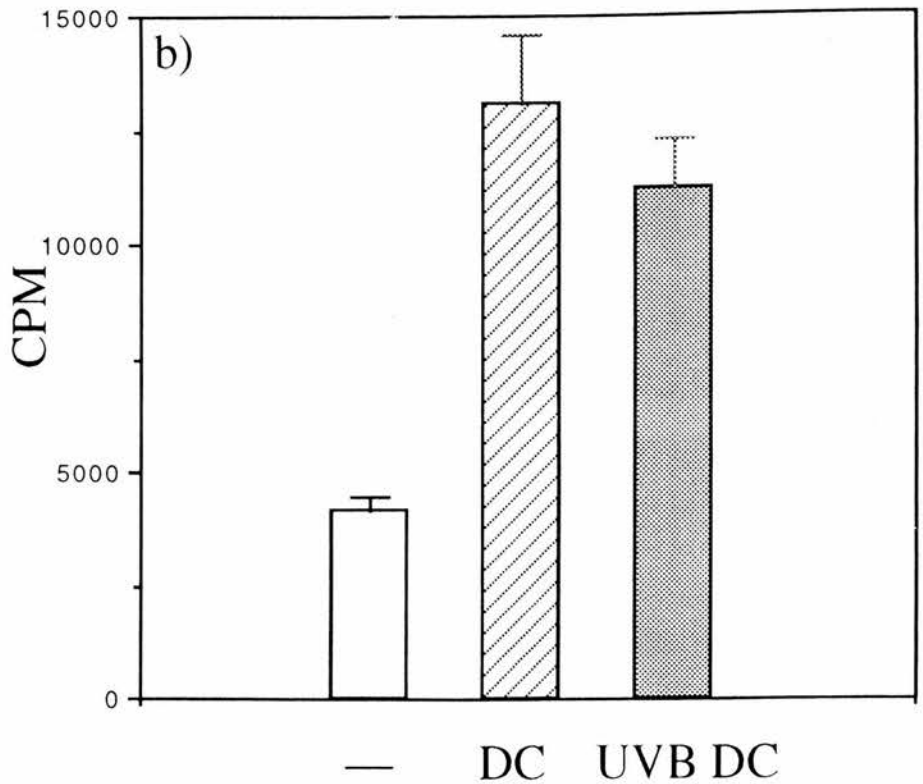
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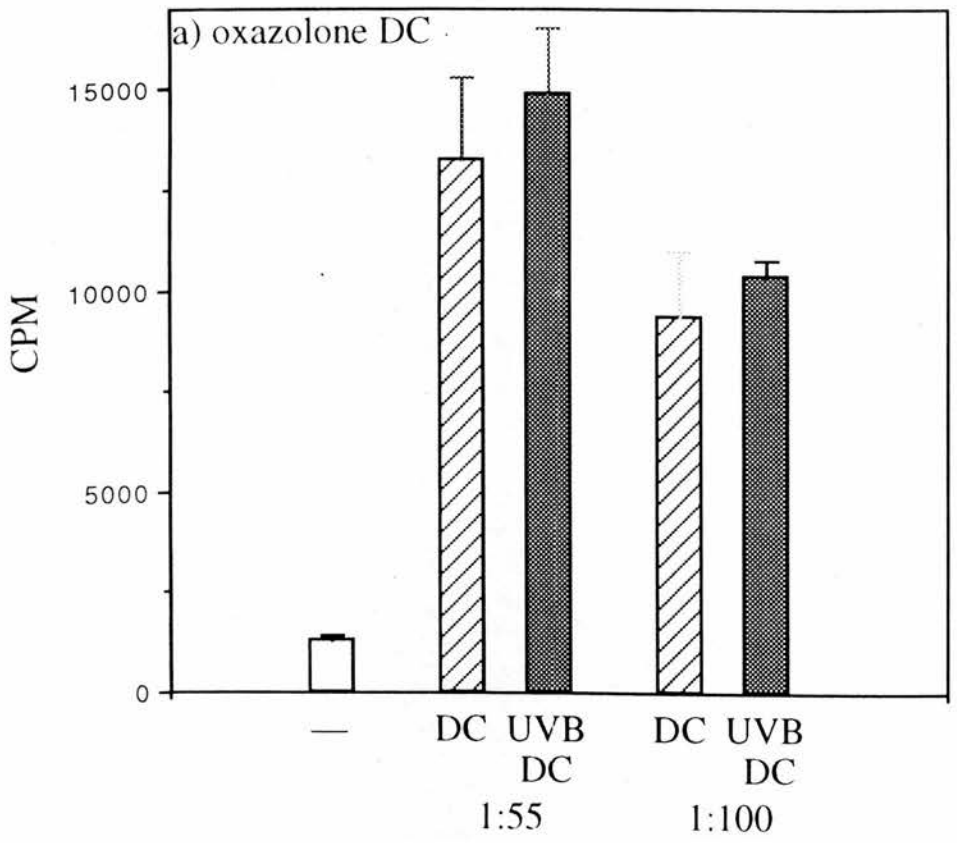
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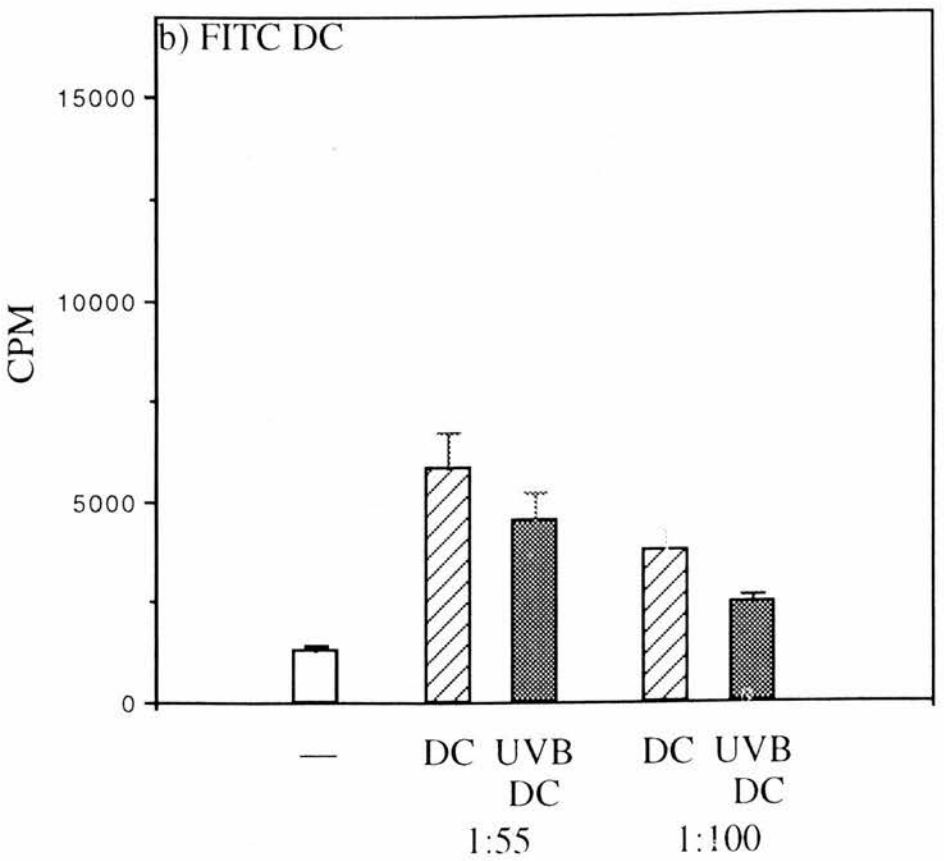
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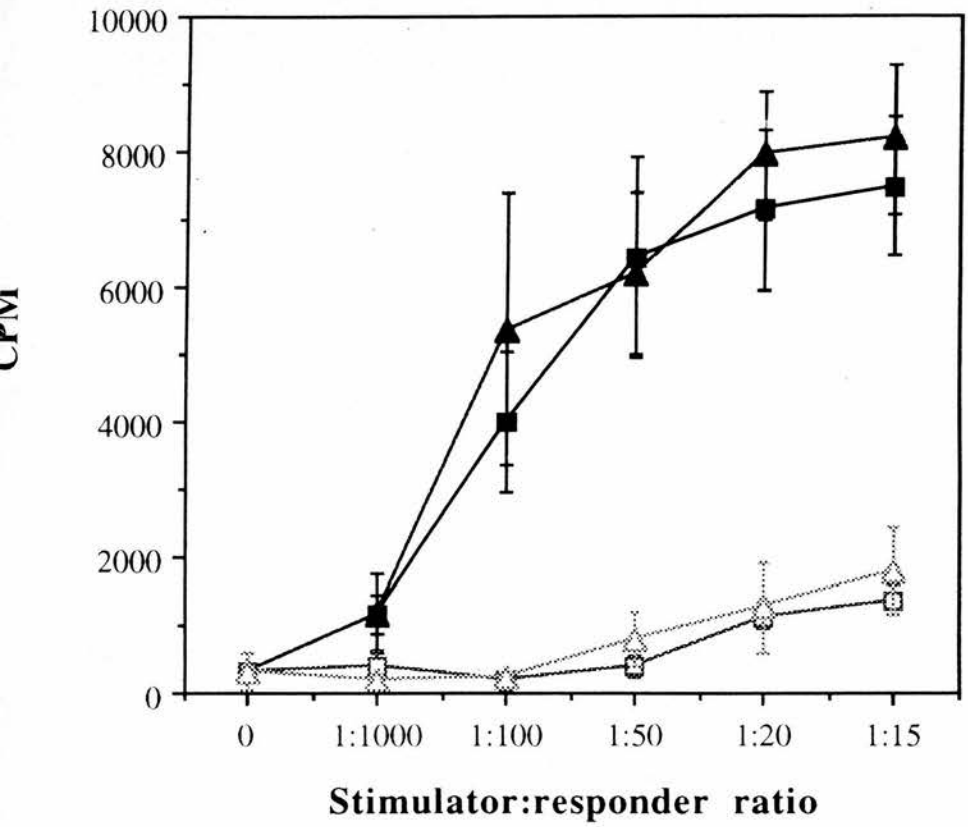
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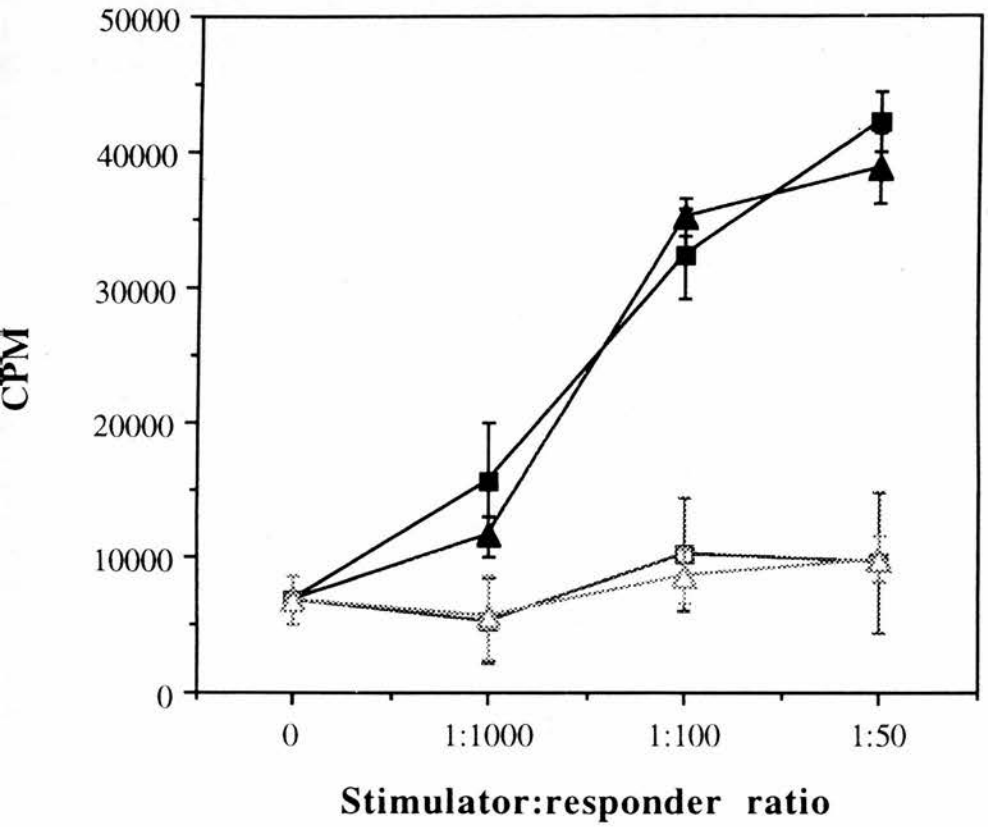
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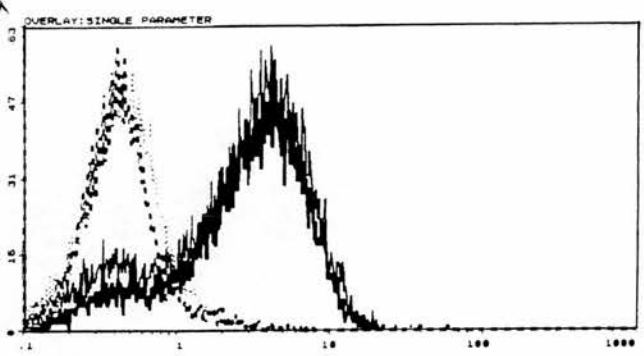
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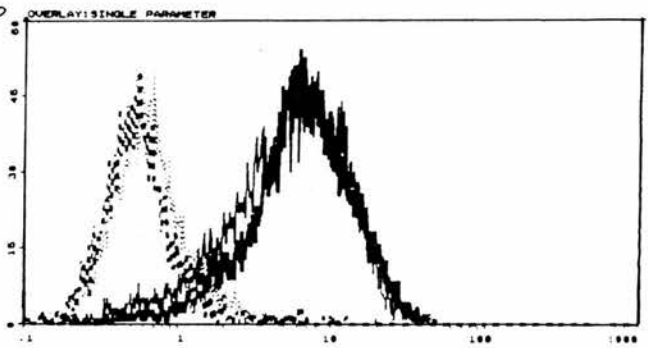
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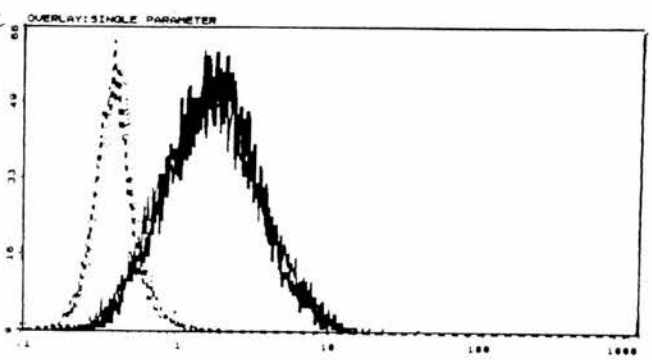
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STRAIN VARIATIONS IN INTERLEUKIN-6 PRODUCTION FOLLOWING SKIN SENSITISATION OF MICE.

Michael B. Lappin, Rebecca J. Dearman[1], Mary Norval, Ian Kimber[1],

Department of Medical Microbiology, University of Edinburgh Medical School, Edinburgh, EH8 9AG, UK. [1] Zeneca, Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire SK10 4TJ, UK.

Correspondence: Dr. M. Norval, Department of Medical Microbiology, University of Edinburgh Medical School, Teviot Place, Edinburgh, Scotland, EH8 9AG.
Tel: 0131 650 3167
Fax: 0131 650 6531

SUMMARY

The ability of draining lymph node cells (LNC) from BALB/c and C3H/HeN mouse strains to elaborate interleukin-6 (IL-6) *in vitro* following topical exposure to the contact sensitiser, oxazolone, has been compared. Consistent with previous investigations it was found that during 24 hr culture LNC prepared from sensitised BALB/c mice expressed high levels of IL-6 (around 8-10ng/ml); the main or exclusive source of which is believed to be dendritic cells (DC). In contrast, draining LNC from C3H/HeN mice exposed in an identical way to oxazolone produced significantly lower levels of IL-6 (≈0.3ng/ml), just above the limits of detection for the assay (0.15ng/ml). The LNC proliferative responses and interferon-γ (IFN-γ) production *in vitro* were comparable in both strains. The lack of IL-6 production by C3H/HeN mice was not due to a lack of DC within LNC populations as similar numbers of DC accumulated in the draining lymph nodes (DLN) of both strains following sensitisation. The differences in IL-6 production in the DLN of C3H/HeN and BALB/c mice did not reflect congenital variation between these strains with respect to IL-6 production *per se* as similar levels of spontaneous and inducible expression of this cytokine in the skin were measured in BALB/c and C3H/HeN mice following, respectively, topical exposure to vehicle alone or to oxazolone. In addition, when LNC from oxazolone treated mice were disrupted by sonication, more IL-6 was released into the supernatant by LNC from C3H/HeN mice (≈2.2ng/ml), than by BALB/c LNC (≈1ng/ml). The concentration of intracellular IL-6 in C3H/HeN LNC fell to 0.7 ng/ml after *in vitro* culture for 24 hrs, while in BALB/c the concentration was maintained at similar levels. These data indicate significant strain variations in terms of the ability of draining LNC to elaborate IL-6 following immune stimulation. The possible association between such variation and susceptibility to UVB-mediated immunosuppression is discussed.

INTRODUCTION

The induction phase of allergic contact dermatitis follows the exposure of susceptible individuals to a sensitising chemical and results in the appearance of an antigen specific effector T cell population in the periphery. After topical application of a sensitising chemical to the skin, haptenated endogenous proteins are processed and immunogenic epitopes displayed on the surface of Langerhans cells (LC) in the epidermis. Changes induced in the epidermal microenvironment by exposure to the chemical allergens causes LC, some of which bear antigen, to migrate from the epidermis and into the afferent lymphatics. The LC then move to the draining lymph nodes (DLN) where they accumulate as immunostimulatory dendritic cells (DC) [1,2]. During migration LC undergo changes in phenotype and function. They become efficient APC and acquire the ability to cluster with and activate naive antigen-responsive T cells [3]. This ability to induce the proliferation and differentiation of naive T cell clones into antigen-specific effector T cells is vital for the induction of primary immune responses.

Interleukin-6 (IL-6) is a cytokine produced by a wide variety of cells in response to injury, infection and a range of cytokines. It acts on a number of cell populations and tissues, with its main effects being the stimulation of growth and differentiation of B and T lymphocytes, the initiation of acute phase protein production by hepatocytes and the induction of responsiveness to Interleukin-3 (IL-3) in bone marrow stem cells [4]. The induction phase of skin sensitisation in BALB/c mice is characterised by the stimulation of proliferative responses in lymph nodes draining the site of exposure and an associated production of IL-6 [5]. The DC that accumulate in the DLN following sensitisation represent the predominant source of this cytokine [6]. These DC are central to the initiation of immune responses and their ability to produce IL-6 is

of interest because of the role of this cytokine in inducing (interleukin-2) IL-2 responsiveness in T lymphocytes [4].

Since IL-6 is important in the early stages of T cell activation, the purpose of these investigations was to investigate the relationship between IL-6 production by lymph node cells (LNC) *in vitro*, the proliferation of these LNC and DC accumulation in the lymph nodes of two strains of mice following contact sensitisation. The two strains chosen were C3H/HeN and BALB/c that show, respectively, susceptibility and resistance to the immunosuppressive effect of UVB on CH responses [7,8].

MATERIALS AND METHODS

Animals

Female C3H/HeN and BALB/c strains of mice, aged 6-8 weeks, were obtained from the specific pathogen-free animal breeding facility at the Medical Microbiology Transgenic Unit, University of Edinburgh.

Contact sensitiser

Oxazolone (4-ethoxymethelene-2-phenyloxazol-5-one) was obtained from Sigma Aldrich Co. (Poole, UK). It was used at 1% w/v in 4:1 acetone:olive oil (AOO).

Enrichment of dendritic cells

The method of Macatonia et al [2] was followed in outline. DC were enriched from the pooled peripheral lymph nodes (auricular, auxillary, inguinal and popliteal) of untreated mice (n=6-10) or from pooled auricular lymph nodes of mice (n=10-15) 18 hrs after ear painting with 25µl of 1% oxazolone. A single cell suspension of LNC was prepared by mechanical dissagregation through 200 mesh stainless steel gauze (J. Stanier & Co. Manchester, UK), viable cells were counted by exclusion of 0.5% trypan blue and were resuspended in RPMI-1640 (Gibco BRL, Paisley, Scotland) supplemented with 25mM HEPES, 100 i.u./ml penicillin, 200µg/ml streptomycin, 100µg/ml gentamicin, 2mM L-glutamine and 10% heat-inactivated foetal calf serum (FCS) (RPMI-FCS). DC-enriched populations were prepared by density gradient centrifugation on Metrizamide (Nygaard, Oslo, Norway). Briefly, LNC were adjusted to $5x10^6$ cells/ml in RPMI-FCS and 8ml of the cell suspension were gently underlayed with 2ml of 14.5% Metrizamide in RPMI-FCS and centrifuged for 20 minutes (600g) at room temperature. The low buoyant density population (DC^+) that accumulated at the interface was collected and washed twice with RPMI-FCS. Cells with DC morphology (large granular cells with dendrites) were counted using a hemocytometer and the results were

expressed as the number of DC per lymph node± standard deviation (SD) from 5 separate counts.

IL-6 and Interferon-γ (IFN-γ) production and proliferation of draining lymph node cells

Groups of mice (n=5) received either 25μl of oxazolone or an equal volume of vehicle alone (n=10) on the dorsum of both ears. At various times thereafter (2, 3 and 5 days), mice were killed and their auricular lymph nodes excised and pooled for each experimental group. Single cell suspensions of LNC were prepared by mechanical dissaggregation as described previously, cells were washed and resuspended in RPMI-FCS. Viable cell counts were performed by exclusion of 0.5% trypan blue. Cells were resuspended at $1x10^7$ per ml in RPMI-FCS and seeded into separate plates for measurement of cytokine production and LNC proliferation. To analyse cytokine production, 1ml of cell suspension from each group was cultured for 24 or 48 hours in 24-well tissue culture plates (Costar, Cambridge, Ma.). Culture was terminated by centrifugation and the supernatants were stored at -70°C until analysis as described below. To measure proliferation, 5 aliquots of 200μl of LNC at $1x10^7$ cells/ml were seeded into 96-well round bottomed tissue culture plates (Nunc, Roskilde, Denmark). Cells were cultured for 24 hours with 1μCi/well ^{3}H-methyl thymidine (^{3}H-methyl thymidine, 5mCi/5ml. Amersham International plc, UK.). The cultures were terminated by automatic harvesting and ^{3}H-methyl thymidine incorporation measured as counts per minute (CPM) using a liquid scintillation analyser (Canberra Packard, Zurich, Switzerland). The data are presented as the mean CPM±SD from 5 replicate cultures.

Intracellular IL-6 content of draining LNC

Mice received either 25μl of oxazolone (n=10-15) or an equal volume of vehicle alone (n=10-15) on the dorsum of both ears. Three days later groups of mice

were killed and their auricular lymph nodes excised and pooled. Single cell suspensions were prepared as above and cells resuspended at $2x10^8$ LNC in 1ml of RPMI/FCS. The cell suspensions were put on ice and sonicated for 3x10 second cycles (Soniprep 150). Finally, the suspensions were microfuged at $1.5x10^4$g for 5min, the supernatant was collected and stored at -70°C until analysis for IL-6 by enzyme linked immunosorbent assay (ELISA). In some experiments the cells were cultured for 24 hrs in RPMI-FCS at $1x10^7$ cells/ml. Following this period in culture the cells were pooled and washed, before being resuspended at $2x10^8$ cells/ml and sonicated as described above.

Kinetics of cytokine production in the skin

The backs of mice were shaved 24 hours prior to sensitisation. Mice were treated with 100µl of oxazolone, the same volume of AOO or were untreated. Groups of mice (n=4) were killed 0, 1, 2, 4, 8 or 24 hrs later, and samples of skin weighing between 0.04 and 0.1g were collected from the treated site. Each sample was chopped finely, weighed and resuspended in 1ml of RPMI-FCS medium in a 1.5ml eppendorf (Alpha Laboratories, Eastleigh, UK). Each eppendorf was snap frozen in liquid nitrogen, thawed, and the contents were homogenised with a tissue grinder. The homogenised sample was snap frozen again, thawed and the contents sonicated for 10 seconds (Soniprep 150, Sanyo). Debris was removed by centrifugation (5min at $1.5x10^4$g) and the supernatant assayed for IL-6 by ELISA. The results are expressed as nanogram of cytokine per gram of original tissue (ng/g).

Measurement of IL-6 by cytokine-specific ELISA

IL-6 concentration was analysed using a sandwich ELISA described previously [9]. Briefly, plastic microtitre plates (Nunc, Copenhagen, Denmark) were coated overnight at 4°C with 50µl/well of 2.5 µg/ml rat monoclonal anti-mouse-IL-6 (Genzyme, West Malling, UK) diluted in 0.1M carbonate buffer (pH 9.6). Plates were blocked with 100µl/well of 10% FCS in phosphate-buffered saline (PBS; pH 7.2) and incubated at 37°C for 30 minutes. Following the blocking step the recombinant murine IL-6 (Genzyme) was serially diluted in RPMI-FCS and 100 µl were added to triplicate wells. Sample supernatants were also plated out in triplicate (100µl/well) and the plates incubated at room temperature (RT) for 2hrs. Next, 100µl/well of goat anti-murine-IL-6 (R&D Systems, Abingdon, UK) at 8µg/ml diluted in RPMI-FCS were added to each well and the plates incubated at RT for 2hrs. The plates were then incubated for 2hrs at RT with 100µl/well of 1:500 dilution of donkey anti-goat IgG/horseradish peroxidase conjugate (Serotec, Oxford, UK) in RPMI-FCS. Following each incubation, the plates were washed three times with PBS containing 0.05% Tween 20. The enzyme substrate (o-phenylenediamine dihydrochloride and urea hydrogen peroxide) was added. The enzyme reaction was stopped after 15 minutes by addition of 50µl/well of 0.5M citric acid. The optical density (OD) of each sample was measured using a plate reader (Dynatech, UK) at 450nm and a standard curve plotted using the OD_{450nm} against the concentration of IL-6 in ng/ml on a log scale. The standard curve was used to calculate the concentration of IL-6 in ng/ml ± SD in the triplicate samples. The limit of detection of IL-6 was 0.15ng/ml.

Measurement of IFN-γ

IFN-γ was analysed by ELISA, using a method described previously [10]. Briefly, plastic microtitre plates (Nunc, Copenhagen, Denmark) were coated overnight at 4°C with 100µl/well of 0.5µg/ml rat anti-murine-IFN-γ (Genzyme) diluted in 0.1M carbonate buffer (pH 9.6). Plates were blocked with 100µl/well of 5% FCS in PBS and incubated at 37°C for 30 minutes. Following the blocking step the recombinant murine IFN-γ (Genzyme) was serially diluted in RPMI-FCS and 100 µl were added to triplicate wells. Sample supernatants were also plated out in triplicate (100µl/well) and the plates incubated at room temperature (RT) for 2hrs. Next, 100µl/well of goat anti-murine-IFN-γ (Genzyme) at 8µg/ml diluted in RPMI-FCS were added to each well and the plates incubated at RT for 2hrs. The plates were then incubated for 2hrs at RT with 100µl/well of 1:500 dilution of donkey anti-goat IgG/horseradish peroxidase conjugate (Serotec, Oxford, UK) in RPMI-FCS. Following each incubation, the plates were washed three times with PBS containing 0.05% Tween 20. The final steps were as described above for IL-6. The limit of detection for IFN-γ was 0.15 ng/ml.

UV Source and exposure

Mice (C3H/HeN and BALB/c) were irradiated under two Philips TL-20W/12 bulbs with an output range of 270-350nm, peak 305nm, emitting 80mW/ cm^2. The output of this source was determined using a filtered photodiode meter, calibrated against measurements made with a UV-visible spectroradiometer (model 742, Optronic Laboratories Inc.) across the spectral range 250-400nm. The tube to target distance was 16 cm. One minimal erythemal dose for C3H/HeN mice under these conditions was 1500 J/m^2.

Measurement of contact hypersensitivity responses

To measure the effect of UVB on CH responses the shaved backs of mice were exposed to two doses of 1440 J/m^2 UVB, 48 and 24 hours prior to sensitisation. The irradiated group and one of the unirradiated control groups were sensitised on the back with 50µl 1% oxazolone. The backs of the final group were painted with 50µl of vehicle alone (AOO). Five days later the ears of each mouse were measured using an engineers micrometer (Draper SM-510, Japan) and challenged by epicutaneous application of 25µl of 0.25% oxazolone in vehicle on the dorsum of each ear. Twenty four hours later the ears were measured again, the increase in ear thickness calculated and the results expressed as the mean increase of ear thickness for the group in mm^{-2} ± standard error.

The effect of UVB on IL-6 production and intracellular IL-6 levels

Groups of mice (n=5) were exposed to a single dose of 1440 J/m^2 UVB using the UVB source described above, or were not irradiated (n=3). One, three and five days later the auricular lymph nodes from irradiated animals and the peripheral lymph nodes from unirradiated animals were collected and pooled for each group. Proliferation, IL-6 production and intracellular IL-6 content were measured as described previously.

Statistics

The Mann-Whitney test was used to analyse differences between groups. These were considered significant if $p \leq 0.05$.

RESULTS

Proliferation and IL-6 production by LNC *in vitro*

In both C3H/HeN and BALB/c strains, the kinetics of oxazolone induced proliferation were similar, peaking at 2-3 days after sensitisation and decreasing by 5 days (Fig 1a and b). Peak responses were around 65000 CPM for C3H/HeN mice and 38000 CPM for BALB/c mice. Despite exhibiting vigorous proliferation, LNC from C3H/HeN mice produced very small amounts of IL-6; 0.3-0.4 ng/ml on days 2 and 3, and below the limits of detection (<0.15 ng/ml) by day 5 after sensitisation (Fig 1a). In contrast, while LNC from BALB/c mice produced low levels of IL-6, 0.3 and <0.15 ng/ml on day 2 and 5 respectively, they produced comparatively large amounts of IL-6 (8-10 ng/ml) on day 3 after ear painting with oxazolone (Fig 1b). Proliferative responses were below 5000 CPM and the concentration of IL-6 in supernatants was below detectable levels (0.15 ng/ml) at all times following vehicle treatment (data not shown).

This experiment was performed with similar results on four separate occasions. The data for the day 3 timepoint are presented in Table 1. It can be seen that the LNC proliferative responses induced by oxazolone were highly variable in both strains (20000-70000 CPM). However the mean proliferative responses for both strains were around 40000 CPM and there was no significant inter-strain difference. There was considerably less variability in cytokine levels, with C3H/HeN mice producing low to undetectable levels and BALB/c mice producing significantly ($p \leq 0.025$) higher amounts of IL-6 in each of the 4 independent experiments. Similar levels of IL-6 production following oxazolone sensitisation of BALB/c mice have been reported previously [5].

IFN-γ production by LNC

To determine whether the strain differences in IL-6 secretion were a reflection of reduced cytokine production *per se* in C3H/HeN mice, the production by sensitised LNC of IFN-γ was examined. The kinetics of IFN-γ production by LNC were similar to those for IL-6 production, with peak levels 2-3 days following sensitisation. C3H/HeN mice produced 0.66 and 0.58 ng/ml of IFN-γ 2 and 3 days respectively following oxazolone sensitisation. At the same time points BALB/c LNC produced 1 and 1.06 ng/ml of the cytokine. By day 5 following oxazolone sensitisation the IFN-γ production by both strains was below detectable levels (0.15 ng/ml). Table 2 shows C3H/HeN and BALB/c LNC proliferative responses and IFN-γ production 3 days after oxazolone sensitisation in three separate experiments. There was no significant differences between strains with respect to IFN-γ levels in the LNC supernatants, with C3H/HeN mice producing a mean of 0.65±0.13 and BALB/c mice producing 0.95±0.42 ng/ml of IFN–γ. Therefore, unlike IL-6, IFN-γ production by LNC was similar for both C3H/HeN and BALB/c mice.

DC numbers in naive and sensitised lymph nodes

Since IL-6 produced by allergen-activated LNC populations is derived predominantly from DC [6], it was decided to examine DC numbers in the draining lymph nodes of C3H/HeN and BALB/c mice following sensitisation with oxazolone. In both strains, treatment with oxazolone induced a comparable increase in DC numbers in the DLN 18 hrs later. In C3H/HeN mice DC numbers increased from a mean of 2759 to 23479 per node, whereas in BALB/c mice identical treatment caused an increase in mean DC numbers from 2127 to 22920 per node.

IL-6 content of oxazolone-sensitised LNC

Immediate sonication of LNC taken from both strains 3 days after sensitisation with oxazolone resulted in detectable levels of IL-6 in the supernatant (Fig 2). IL-6 levels were higher in LNC from sensitised C3H/HeN and BALB/c mice, compared with LNC from vehicle-treated controls. In addition, sonication of LNC from sensitised C3H/HeN mice resulted in higher levels of IL-6 being released into supernatant than were released from BALB/c LNC after identical treatment.

In order to investigate further the strain differences in IL-6 secretion, C3H/HeN and BALB/c mice were sensitised with oxazolone and 3 days later LNC were prepared and cultured for 24 hrs. Again, there was a marked difference in the secretion of IL-6 by the two strains, with IL-6 being below the detectable limits in the supernatants from C3H/HeN LNC (<0.15 ng/ml) and present at high concentrations (4.3 ng/ml) in the supernatants from BALB/c LNC. Sonication of the cells after 24 hrs released 0.72 and 2.38 ng/ml of IL-6 into the supernatant from C3H/HeN and BALB/c LNC respectively.

Cutaneous IL-6 production by C3H/HeN and BALB/c mice

IL-6 is produced constitutively by epidermal LC [11] and keratinocytes can be induced to synthesise IL-6 [12]. Fig 3 shows that similar amounts of IL-6 were produced constitutively in the skin by both C3H/HeN (3.83±0.97 ng/g of tissue±SD, n=4) and BALB/c mice (3.37±0.58 ng/g of tissue±SD, n=4). Painting the skin of C3H/HEN or BALB/c mice with vehicle did not cause a substantial increase in IL-6 production over constitutive levels. In C3H/HeN mice, sensitisation with 1% oxazolone induced significant increases (when compared with vehicle controls) in IL-6 levels ($p \leq 0.025$) at all timepoints except 0 hr. In BALB/c mice, oxazolone induced a significant increase ($p \leq 0.025$) in IL-6 levels compared to the vehicle control at all timepoints. The kinetics, but not the magnitude, of IL-6 production were similar in both strains, with IL-6 levels in each instance peaking 4-8 hours post-sensitisation and

decreasing by 24 hrs (Fig 3). C3H/HeN mice produced higher levels of IL-6 following sensitisation than did BALB/c mice. At the peak 4 hour timepoint, skin from C3H/HeN mice contained almost twice as much IL-6 than skin from BALB/c mice (36 ng/g and 20 ng/g tissue respectively). This experiment has been repeated on a separate occasion for each strain with similar results.

Suppression of CH responses by low-dose UVB

C3H/HeN and BALB/c mice have been characterised as being, respectively, UVB-resistant and susceptible in a number of different studies using a variety of UVB exposures and CH regimens [8,13]. To confirm that these mice display consistent differences in susceptibility to UVB in our hands, we exposed the backs of mice to 2x1440 J/m^2 of broadband UVB 48 and 24 hrs prior to sensitisation on the irradiated site with oxazolone. The irradiation resulted in a significant ($p=0.05$) 31% reduction in ear swelling in C3H/HeN mice challenged with oxazolone (Fig 4a). In Fig 4b it can be seen that exposure of BALB/c mice to the same dose of UVB failed to cause a significant suppression of contact hypersensitivity. Therefore, using the standard immunosuppressive protocol employed here, C3H/HeN mice were found to be susceptible and BALB/c mice resistant to the local suppression of CH responses by UVB.

IL-6 production and intracellular levels of IL-6 following UVB

Exposure to UV has been reported previously to result in the accumulation of DC in the DLN [14]. In the present study, exposure to a single dose of 1440 J/m^2 of UVB caused a comparable increase in DC numbers in DLN of both strains 18 hrs later. In C3H/HeN mice DC numbers rose from 2083 to 7524 DC/LN, and from 2530 DC/LN to 6223 DC/LN in BALB/c mice.

Though UVB caused an accumulation of DC in the DLN, LNC from lymph nodes draining irradiated skin failed to produce appreciable levels of IL-6 in culture, and had low levels of intracellular IL-6, irrespective of strain (data not shown).

DISCUSSION

Here we report a marked strain difference with respect to the ability of LNC to secrete IL-6 after skin sensitisation. LNC from BALB/c mice produced high levels of IL-6 *in vitro* with IL-6 levels peaking 3 days after sensitisation. In contrast, draining LNC from C3H/HeN mice secreted low or undetectable levels of IL-6 when sensitised identically. Proliferative responses and IFN-γ secretion by both strains were comparable, suggesting either that IL-6 availability is not an absolute requirement for LNC activation, or that the low concentrations of IL-6 secreted in culture by C3H/HeN LNC give sufficient co-stimulation for T cell activation.

The kinetics of IL-6 production in the skin after treatment with oxazolone was also investigated. Previous studies using contact allergens and irritants have shown that IL-6 is induced by contact allergens but not irritants, when these chemicals are used at doses which cause comparable inflammatory responses [15]. Similar levels of IL-6 were produced constitutively in the skin of both C3H/HeN and BALB/c mice. In both strains oxazolone treatment induced significant increases in IL-6 production peaking 4 hours after sensitisation. Therefore the lack of IL-6 secretion by sensitised LNC from C3H/HeN mice does not reflect a systemic inability to produce IL-6. Both keratinocytes and LC are able to produce IL-6 [11,12]. This study has not addressed whether LC in the skin of C3H/HeN mice have the ability to produce IL-6, as has been reported for LC from BALB/c mice [16].

DC are the main source of IL-6 within LNC populations. Complement-depletion of DC from oxazolone-sensitised LNC suspensions reduces IL-6 activity by more than 75% but does not affect proliferative responses [6]. If the same method is used to deplete T cells, the proliferation but the not IL-6 production by LNC *in vitro* is affected. Because DC are the main source of IL-6 within lymph nodes, the number of DC that accumulated in the DLN of both strains following sensitisation was examined. Treatment with oxazolone

resulted in the accumulation of similar numbers of DC in both C3H/HeN and BALB/c mice. Therefore the lack of IL-6 production in C3H/HeN mice does not reflect a paucity of DC in the DLN.

LNC from C3H/HeN and BALB/c mice were sonicated to examine strain differences in the intracellular levels of IL-6. Sonicating LNC immediately after removal from oxazolone treated C3H/HeN and BALB/c mice caused the release of appreciable quantities of IL-6 into supernatants, while sonication of LNC from mice treated with the vehicle alone resulted in less IL-6 activity in the supernatant, suggesting that LNC from both strains are capable of translating IL-6 protein. The inability of oxazolone-sensitised LNC from C3H/HeN mice to secrete IL-6 *in vitro* may be associated with a lack of IL-6 production, or may reflect a failure in the ability of cells to secrete IL-6. To investigate this, oxazolone-sensitised LNC from C3H/HeN and BALB/c mice were cultured for 24 hrs and the concentration of IL-6 in the supernatants and sonicates was measured. The inability of C3H/HeN mice to secrete IL-6 in culture was not associated with an accumulation of intracellular IL-6. Therefore it is likely that the low levels of IL-6 produced by C3H/HeN LNC reflect a lack of IL-6 production by LNC, rather than a failure in IL-6 secretion.

C3H/HeN and BALB/c mice show, respectively, susceptibility and resistance to the immunosuppressive effect of UVB on CH responses [8]. In C3H/HeN mice exposure to 1440 J/m^2 of UVB 48 and 24 hours prior to sensitisation caused a 31% suppression of CH responses to oxazolone. In our hands CH responses were not suppressed in BALB/c mice following exposure to the same dose of UVB. The basis for this strain difference is unclear, but UVB may act by altering the DC population that migrates from irradiated skin to the DLN. This being the case it is interesting that DC from C3H/HeN and BALB/c mice display such marked differences in their ability to produce IL-6 following sensitisation.

In agreement with previous studies [17], we found UVB-induced accumulation of DC in the local DLN of C3H/HeN mice and in addition

recorded comparable numbers of DC in the DLN of BALB/c mice following UVB. However, at least in the BALB/c strain, these DC differ substantially from those which migrate to the DLN after contact sensitisation. While LNC from sensitised BALB/c mice produce high levels of IL-6 and T cell derived IFN-γ, LNC from UVB treated mice fail to produce either cytokine (data not shown for IFN-γ). This suggests that DC migration is not in itself sufficient for all aspects of DC maturation and that an antigen dependent signal is required for IL-6 production by DC and the induction of IFN-γ production by T cells.

In conclusion, we have shown that in C3H/HeN mice oxazolone induced DC accumulation in the DLN but, unlike DC from BALB/c mice, DC from C3H/HeN mice produced extremely low levels of IL-6. The lack of IL-6 production by C3H/HeN LNC did not affect LNC proliferative activity *in vitro*. The marked difference in DC activity following sensitisation is interesting and may result in functional differences in the induction of effector cell populations in these strains.

Further work is required to investigate the association of IL-6 production by DC in the DLN and the susceptibility of these mice to UVB-induced immunosuppression. Initially it would be informative to characterise the ability of DC from other UVB susceptible and resistant strains to produce IL-6 following contact sensitisation. This work would confirm if IL-6 production, or lack of it, in sensitised lymph nodes can be correlated with the resistance/susceptibility to UVB.

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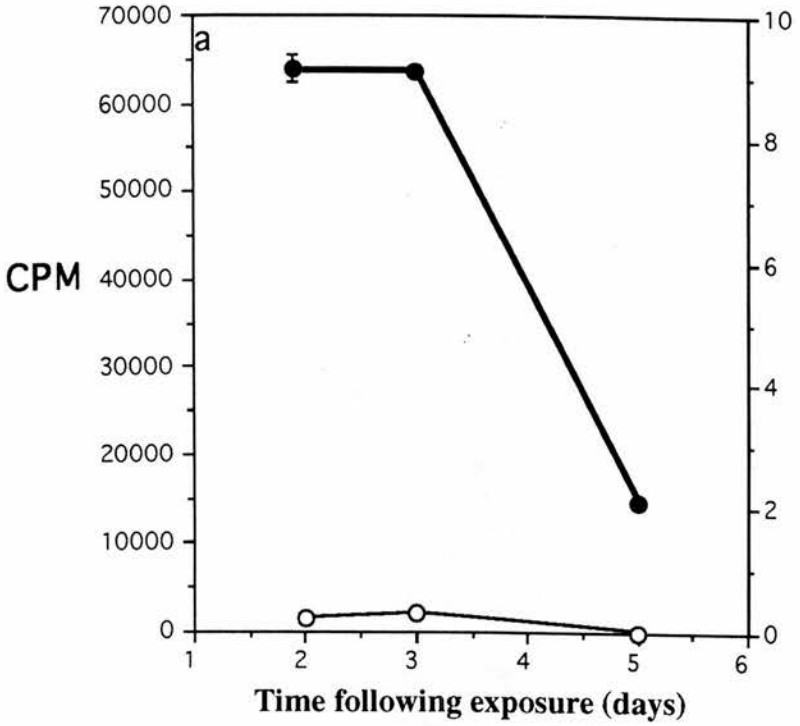
FIGURE LEGENDS

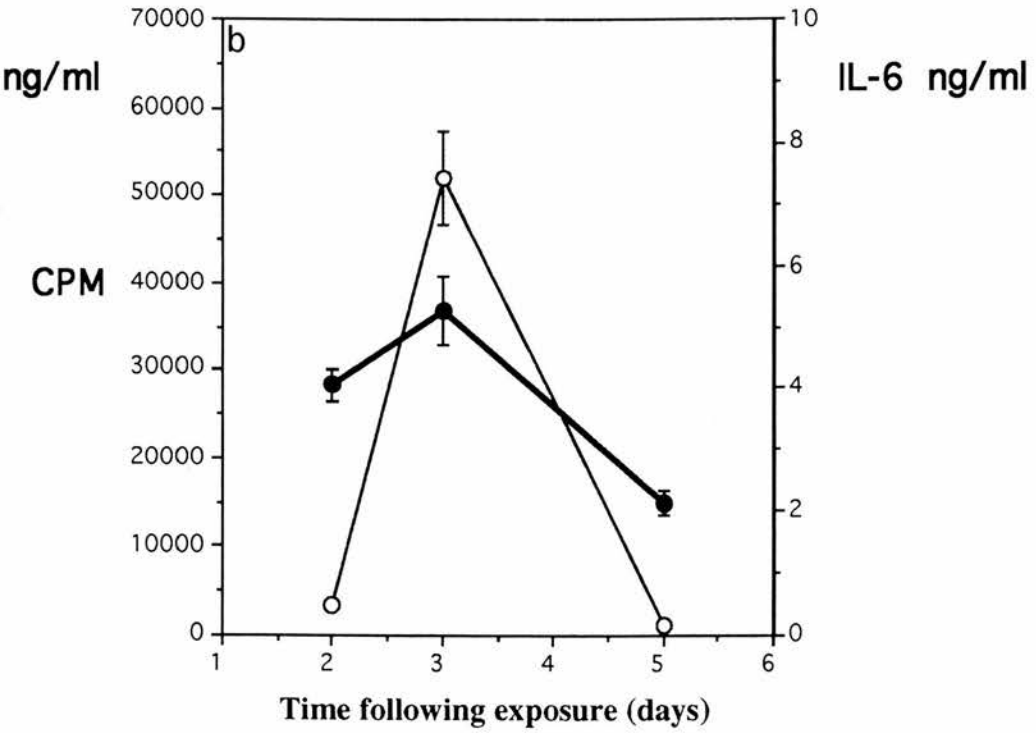
Figure 1. Kinetics of IL-6 production and proliferative activity of C3H/HeN (a) and BALB/c (b) LNC *in vitro*. LNC were taken from oxazolone treated mice and cultured for 24 hours. Proliferative responses in CPM±SD (closed circle) and IL-6 production in ng/ml±SD (open circle) are shown. SD's below 4000 CPM for the proliferative data and below 0.8 ng/ml for the IL-6 data are not shown. Limit of detection for IL-6 = 0.15 ng/ml.

Figure 2. Intracellular IL-6 content of LNC from C3H/HeN and BALB/c mice. The results show IL-6 levels (ng/ml±SD) in supernatants following sonication of LNC. Draining LNC were removed from mice 3d after skin painting with oxazolone (Ox) or vehicle (AOO). IL-6 was below detectable levels in sonicates of C3H/HeN LNC from AOO treated mice.

Figure 3. Kinetics of IL-6 production in the skin of C3H (triangles) and BALB/c mice (circles) after painting with oxazolone (closed) and AOO (open); control (naive) mice were untreated. IL-6 levels are shown as ng per gram tissue±SD. SD's less than 1 ng/g not shown. Limit of detection for IL-6 = 0.15 ng/ml.

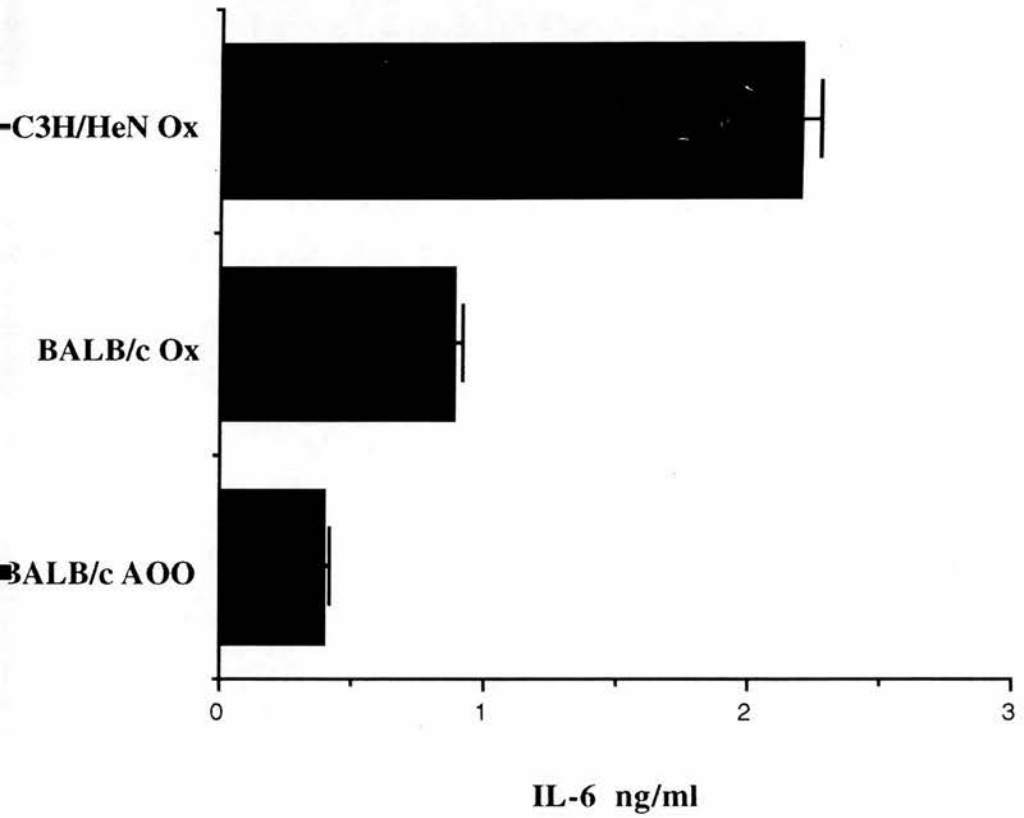
Figure 4. Suppression of CH responses to oxazolone by UVB in C3H/HeN (a) and BALB/c mice (b). Mice were exposed to UVB (1440 J/m^2, -48 and-24 hrs) or were unirradiated, prior to epicutaneous application of vehicle (AOO) or oxazolone (Ox) on the same site. Five days later the ears of all mice were challenged with oxazolone and the results expressed as the mean 24 hour increase in ear swelling in mm^{-2}± SEM, n=7.


a
CPM
IL-6 ng/ml
70000
60000
50000
40000
30000
20000
10000
0
10
8
6
4
2
0
1
2
3
4
5
6
Time following exposure (days)


b
CPM
IL-6 ng/ml
70000
60000
50000
40000
30000
20000
10000
0
10
8
6
4
2
0
1
2
3
4
5
6
Time following exposure (days)

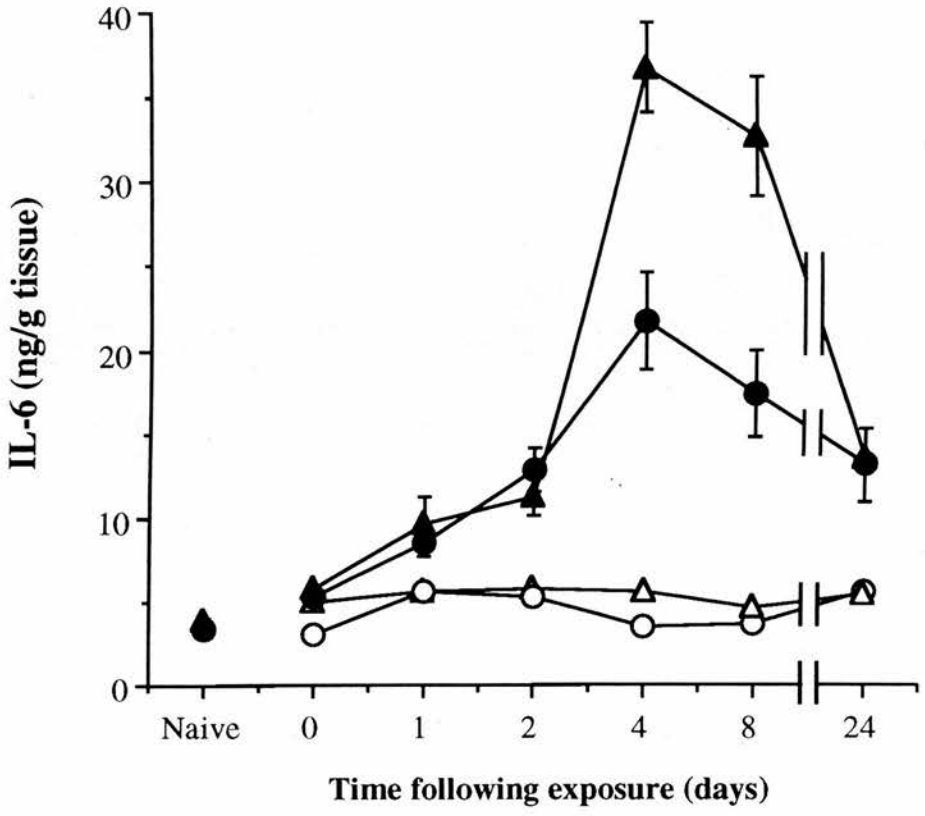
	C3H/HeN		BALB/c	
	Proliferation (CPM)	**IL-6 ng/ml**	**Proliferation (CPM)**	**IL-6 ng/ml**
	66730	0.16	36727	8.00
	22838	0.26	30779	8.10
	63816	0.35	72199	7.10
	22265	0.15	20365	10.5
mean±SEM	43912±12347	0.23±0.05	40018±11242	8.42±0.72

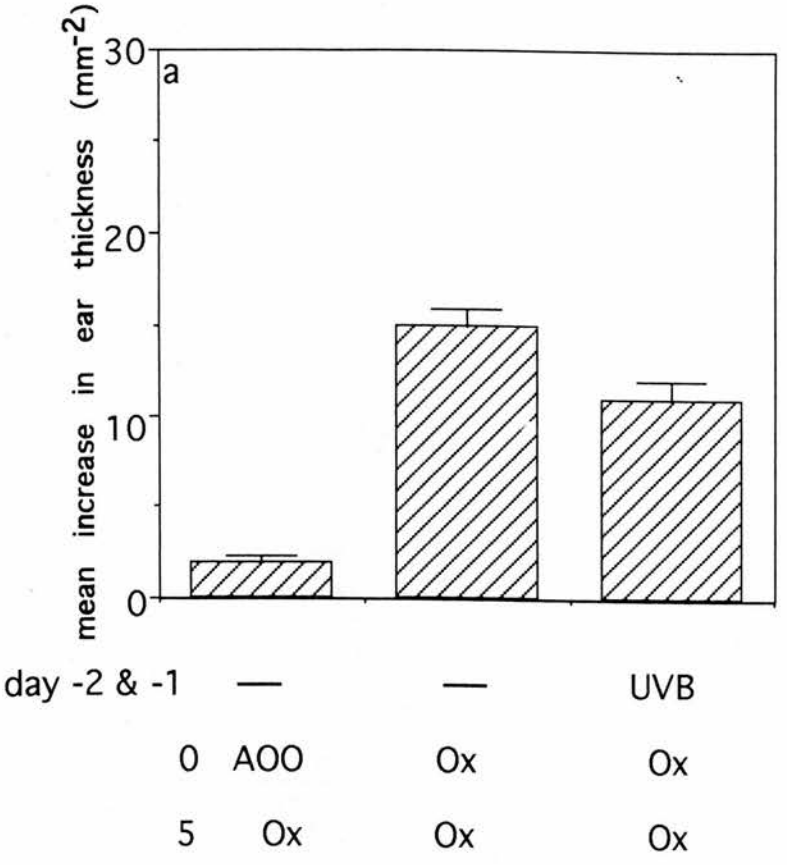
Table 1. Strain differences in LNC proliferative activity and IL-6 production *in vitro* following oxazolone sensitization. Three days after ear painting with oxazolone auricular lymph nodes were removed, single cell suspensions of LNC prepared and cultured for 24 hours. The proliferative activity was analysed by ^{3}H-thymidine incorporation and supernatants were examined for IL-6 activity by ELISA. The mean CPM (n=5) and mean IL-6 (n=3) levels from four separate experiments are shown for each strain. In the final row these figures have been used to provide the mean proliferative response and IL-6 concentration ± SEM for the four separate experiments for both C3H/HeN and BALB/c mice.

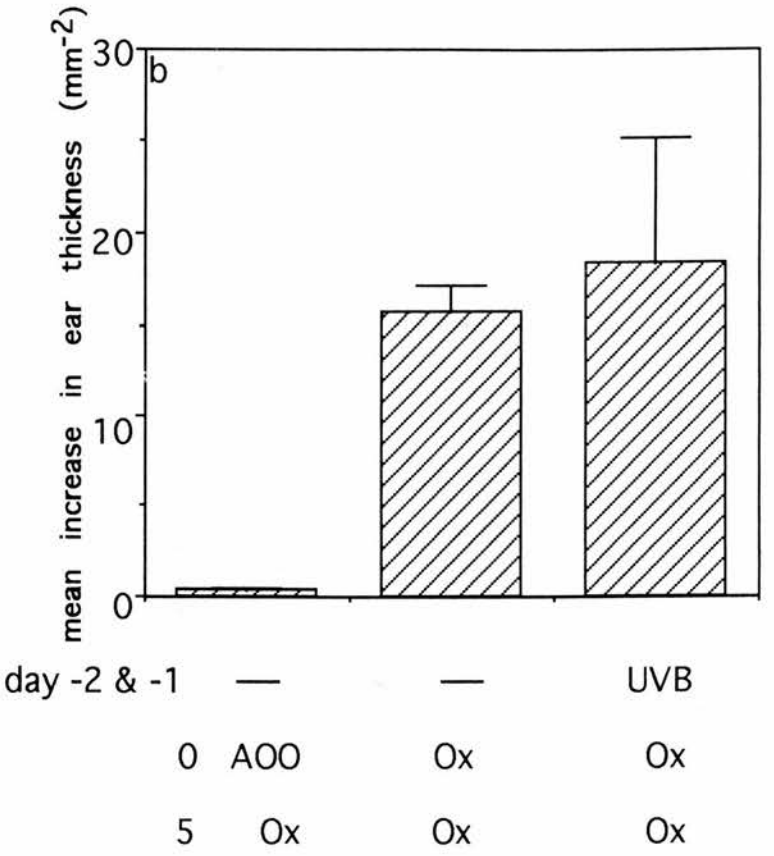

-C3H/HeN Ox
BALB/c Ox
BALB/c AOO
0
1
2
3
IL-6 ng/ml

	C3H/HeN		BALB/c	
	Proliferation (CPM)	**IFN-γ ng/ml**	**Proliferation**	**IFN-γ ng/ml**
	66730	0.80	30779	1.06
	63816	0.58	34613	0.49
	15338	0.57	72199	1.31
mean±SEM	48628±16666	0.65±0.08	45864±13214	0.95±0.24

Table 2 Strain differences in LNC proliferative activity and IFN-γ production *in vitro* following oxazolone sensitization. Three days after ear painting with oxazolone, auricular lymph nodes were removed, single cell suspensions of LNC were prepared and cultured for 24 hours. The proliferative activity was analysed by ^{3}H-thymidine incorporation and supernatants were examined for IFN-γ activity by ELISA. The mean CPM (n=5) and mean IFN-γ (n=3) levels from three separate experiments are shown for each strain. In the final row these figures have been used to provide the mean proliferative response and IFN-γ concentration ± SEM for the three separate experiments for both C3H/HeN and BALB/c mice.


IL-6 (ng/g tissue)
40
30
20
10
0
Naive
0
1
2
4
8
24
Time following exposure (days)


a
mean increase in ear thickness (mm-2)
30
20
10
0
day -2 & -1 — — UVB
0 AOO Ox Ox
5 Ox Ox Ox


b
mean increase in ear thickness (mm-2)
30
20
10
0
day -2 & -1 — — UVB
0 AOO Ox Ox
5 Ox Ox Ox

6

THE ROLE OF CIS-UROCANIC ACID IN UVB-INDUCED IMMUNOSUPPRESSION.

M. B. Lappin[1], A. El-Ghorr[1], I. Kimber[2], M. Norval[1].
[1] Edinburgh University, Edinburgh and [2] Zeneca, Macclesfield, U.K.

UV radiation causes many alterations in skin including loss of Langerhans cells (LC) and also results in accumulation of dendritic cells (DC) in the lymph nodes draining the site of irradiation. One epidermal mediator which may be involved in the immunomodulation is cis-urocanic acid (cis-UCA), formed from the naturally occurring trans-isomer on UV exposure. In this study the role of cis-UCA in epidermal LC depletion and DC accumulation in the lymph nodes was examined. Mice were painted on the ears with cis-UCA or UVB irradiated (144 mJ/cm^{-2}) on their shaved backs; in some cases pre-treatment by intraperitoneal injection of a monoclonal antibody with specificity for cis-UCA was carried out 2 h previously. Mice were killed 24 h later. The number of LC in the epidermal sheets prepared from the ears was counted using ATPase staining, and the number of DC in the lymph nodes estimated following metrizamide purification. UVB exposure or cis-UCA caused a substantial reduction in epidermal LC numbers and the depletion was abrogated in both cases by pre-treatment with the anti-cis-UCA antibody. UVB exposure, but not cis-UCA, induced DC accumulation in lymph nodes, and this migration was not affected by the anti-cis-UCA antibody. Thus cis-UCA is an important mediator of some, but not all, the effects of UVB on the immune system.

LANGERHANS CELLS

(7)

7

IL-6 PRODUCTION BY DRAINING LYMPH NODE CELLS IN UVB RESISTANT AND UVB SUSCEPTIBLE STRAINS OF MICE. M.B. Lappin, R.J. Dearman[1] M. Norval, I. Kimber[1]. Department of Medical Microbiology, University of Edinburgh Medical School, Edinburgh. [1]Zeneca, Central Toxicology Laboratory, Alderley Park, Cheshire

The induction phase of skin sensitization in mice is characterized by the stimulation of proliferative responses in lymph nodes draining the site of exposure. Proliferative activity has been shown previously to be associated with the production by draining lymph node cells (LNC) of interleukin-6 (IL-6); the main source of which appears to be dendritic cells. As IL-6 is known to be an important costimulator in T lymphocyte activation, we have compared the production by LNC of this cytokine in two strains of mice, BALB/c and C3H-HeN, which are considered, respectively, to display resistance or susceptibility to ultraviolet B (UVB)-mediated immunosuppression. Mice were treated on the dorsum of both ears with 1% oxazalone (Ox) in 4:1 acetone:olive oil (AOO), or with AOO alone. Three days later draining (auricular) lymph nodes were excised and a single cell suspension of LNC prepared. LNC were cultured for 24 or 48 hours. The concentration of IL-6 (pg/ml) in the supernatants was measured by enzyme-linked immunosorbent assay. Exposure of either strain of mice to vehicle alone failed to stimulate the production of detectable levels (150 pg/ml) of IL-6. Sensitization of BALB/c strain mice with Ox provoked a vigorous proliferative response by LNC (measured by incorporation of radiolabelled thymidine) and high levels of IL-6. In contrast, although LNC prepared from sensitized C3H-HeN mice exhibited comparable proliferative activity, the production of IL-6 was substantially less. These data reveal marked strain differences in the stimulation of IL-6 responses during the induction of skin sensitization and suggest an association between induced levels of this cytokine and susceptibility to UVB-mediated immunosuppression.

Major conditions (see second announcement for details). Abstract will automatically be ejected for technical reasons if they do not comply.

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OP82 CYTOKINE EXPRESSION AND CELL MOBILISATION IN RAT LUNG INFLAMMATION

C.M.M.Williams, *L.Smith, †B.F.Flanagan, *L. S.Clegg and J.W.Coleman
Dept of Pharmacology., †Dept of Immunology., University of Liverpool L69 3BX. and *Research Dept. Knoll Pharmaceuticals. NG2 3AA. Nottingham.

We investigated kinetics of mRNA expression for IL-5, IL-6 TNF-α, MIP-2 and IFN-γ by bronchoalveolar (BAL) cells and patterns of cell infiltration into the bronchoalveolar lumen in a rat model of Sephadex-induced lung inflammation. Semi-quantitative analysis by RT-PCR of RNA extracted from BAL cells from Sephadex treated rats revealed significant increases in cytokine mRNA. IL-5 mRNA peaked at 48-72 hr. IL-6 mRNA peaked at 48 hr. TNF-α mRNA remained elevated from 6 to 72 hr. MIP-2 mRNA peaked at 24hr, and IFN-γ mRNA was elevated from 24-72 hr. Mononuclear cells reached peak values three days after the Sephadex injection whilst neutrophils peaked at 24-48 hr and eosinophils at 72 hr post-treatment. Similarities between the patterns of neutrophilia, eosinophilia and cytokine mRNA expression seen here compared to those observed in human asthma suggests that this model may prove valuable for further investigation of the role of cytokines in inflammatory lung disease.

OP83 INDUCED INTERLEUKIN-6 (IL-6) PRODUCTION IN THE SKIN AND LYMPH NODES OF UVB RESISTANT AND SUSCEPTIBLE MICE.

MB Lappin, RJ Dearman[1], M Norval, I Kimber[1].

Department of Medical Microbiology, University of Edinburgh.
[1] Zeneca, Central Toxicology Laboratory, Alderley Park, Cheshire.

Mice of BALB/c and C3H/HeN strains are considered to be resistant and susceptible respectively to suppression of contact hypersensitivity induced by UVB irradiation. It has been demonstrated previously that topical exposure of BALB/c mice to the contact allergen oxazolone stimulated vigorous IL-6 production (=10000pg/ml) by draining lymph node cells (LNC) on culture in vitro, the major source of the cytokine being dendritic cells. We now report that while exposure of C3H/HeN mice to oxazolone under identical conditions results in proliferative responses of LNC of equivalent magnitude to those observed with LNC from BALB/c mice, IL-6 production was, by comparison, profoundly reduced (= 300pg/ml). These differences do not reflect a generalised strain difference in IL-6 production as BALB/c and C3H mice exhibit similar levels of constitutive and oxazolone-induced IL-6 expression in the skin. The data reveal an interesting association between susceptibility to UVB and IL-6 expression by LNC.

OP84 ANTIGEN-INDUCED T CELL INFLUX AND CHANGES IN CYTOKINE mRNA IN THE BROWN NORWAY RAT LUNG.

S Underwood, D Noble, C Lawrence, M Foster, S Webber and J-A Karlsson.
Rhône-Poulenc Rorer, Dagenham Research Centre, Essex RM10 7XS.

Cytokines secreted by Th2-type $CD4^+$ T lymphocytes are implicated as mediators of allergic airway inflammation in asthma. IL-4 induces IgE class switching and IL-5 promotes airway eosinophilia which is implicated in the pathogenesis of airway inflammation and hyperreactivity.

Brown Norway rats (group size n = 5) were challenged with inhaled antigen (ovalbumin) and inflammatory changes in the lung were studied 24 hours later. There was an influx of $CD4^+$ T cells (+ 64 %, $p < 0.01$) and eosinophils (+ 607 %, $p < 0.001$) into the lung tissue, but no change in the number of $CD8^+$ T cells. mRNA was extracted from lung tissue and reverse transcribed. PCR revealed increased expression of mRNA for IL-4 and IL-5, but not the Th1 cytokine IFNγ. The increase in airway resistance induced by aerosolized acetylcholine (20 nM, 8 sec) was potentiated (+ 16 ± 5 % in unchallenged rats, + 68 ± 15 % in challenged rats, $p < 0.01$) demonstrating that the airways were hyperreactive.

These data suggest that $CD4^+$ T cells of a Th2 phenotype may be involved in the orchestration of allergic airway inflammation in the Brown Norway rat. This model allows further definition of the pro-inflammatory roles of $CD4^+$ T cells in the pathology of asthma.

OP85 RELEASE OF MOUSE TUMOR NECROSIS FACTOR-ALPHA BY MOUSE CENTRAL CORNEA EXPLANTS. DEVELOPMENT OF AN ELISA FOR THE QUANTITATION OF mTNFα.

T. De Kesel, M. Sekine-Okano, R. Lucas, D. Rungger, D. Pollet, G. E. Grau, P. M. Leuenberger, and E. Rungger-Brandle
Innogenetics N.V., Zwijnaarde, Belgium; Electron Microscopy Lab., Dept. Ophthalmology, Geneva, Switzerland; Dept. Pathol., CMU, Geneva, Switzerland; Dept. Animal Biol., Univ. Geneva, Switzerland.

To accurately determine mTNF levels in various biological fluids and cell culture supernatants, a mTNF ELISA was developed. The ELISA uses a rabbit anti-mTNF polyclonal antibody as capturing antibody, and as detection antibody (biotinylated). The detection limit of the assay is 15 pg/ml; the measuring range lies beween 31 and 3000 pg/ml.

The utility of the assay was demonstrated by examining the capability of corneal cells to release mTNF upon lipopolysaccharide (LPS) stimulation and investigating whether mTNF production could be modulated by pharmacological agents. LPS stimulated mTNF release into culture medium. Addition of budesonide or prednisolone inhibited the release of LPS-stimulated mTNF, while cyclosporin A had no effect.

Conclusions. Upon LPS stimulation of epithelial cells from mouse central cornea, mTNF is produced and released into the medium. Corticosteroids, such as prednisolone and budesonide, effectively inhibit mTNF production.

OP86 CRYPTOCOCCAL ENVELOPE ' COMPONENTS INDUCE DIFFERENT TNF-α LEVELS IN HUMAN MONOCYTES.

W. Chaka, J. Scharringa, V. Vaishnav*, R. Cherniak*, A. Verheul, J. Verhoef, H. Snippe and I.M. Hoepelman. Eijkman-Winkler Institute, Utrecht University, The Netherlands, and Georgia State University, Atlanta, Georgia, USA.

Cryptococcus neoformans enhances HIV replication in peripheral blood mononuclear cells (PBMC) probably by the induction of cytokines. Therefore, we studied the ability of whole cryptococci (CN), the capsular polysaccharide glucuronoxylomannan (GXM), and the minor carbohydrate antigens, galactoxylomannan (GalXM) and two manno-protein fractions (MP1 and MP2) to induce TNF-α secretion in PBMC. CN and the purified components were opsonised either with heat-inactivated or non-treated human pooled serum (HPS) and subsequently incubated with PBMC. CN and all the components induced TNF-α as determined by ELISA, but differences between the amount of TNF-α evoked and the required opsonin was observed. MP2 was the most potent TNF-α inducer. TNF-α induction by CN, GXM and GalXM was complement dependent. In contrast, MP2 induced TNF-α when opsonised with either heat inactivated HPS, HPS or agammaglobulinaemic serum. TNF-α production induced by MP2 could be blocked by two anti-CD 14 monoclonal antibodies whilst isotype immunoglobulins did not. These results suggest that possibly the activation by MP2 of PBMC to release TNF-α involves the CD14 receptor.

OP87 INFLUENCE OF DEXAMETHASONE ON ALLERGEN-ACTIVATED LYMPH NODE CELL INTERFERON-γ EXPRESSION

R J Dearman and I Kimber. Zeneca Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, SK10 4TJ.

We have demonstrated previously that draining lymph node cells (LNC) prepared from mice exposed topically to the contact allergen oxazolone secrete substantial amounts of the T helper 1 (T_{H1}) cytokine interferon γ (IFN-γ; measured by enzyme-linked immunosorbent assay) and display significant proliferative activity (analyzed by 24hr incorporation of radiolabelled thymidine). Culture of LNC with 2μg/ml of the T cell mitogen concanavalin A potentiated markedly both thymidine incorporation and IFN-γ expression. We have now examined the influence of the synthetic glucocorticoid dexamethasone on LNC IFN-γ production and proliferation. Under conditions of exposure where proliferative activity was unaffected, dexamethasone (0.1 to 100μM) inhibited profoundly spontaneous and mitogen-augmented IFN-γ production by LNC. These data demonstrate that dexamethasone modulates the secretion *in vitro* of murine IFN-γ in a manner analogous to that reported previously for the production of human IFN-γ *in vivo* and *in vitro*.

the epidermis and marked proliferation and activation of vessels in the ɔep dermis with an inflammatory infiltrate consisting mainly of eosinophilic ranulocytes in BN and mononuclear cells in Lewis and Wistar rats. In BN ıd Lewis rats and less so in Wistar rats, HCB caused a dose-dependent ɔtivation of endothelial cells of lung venules. At the ultrastructural level, ne endothelial cells were characterized by a marked proliferation of Golgi nd RER and resembled high endothelial venules (HEV) in lymph nodes nd were therefore called HEV-like. The observation that HCB causes strain-ependent lesions in skin and lung, makes an immune etiology conceivable.

324 INHIBITORY EFFECTS OF PENICILLIN G (PCG) AND LATAMOXEF (LMOX) ON ANAPHYLACTIC REACTIONS INDUCED BY THEIR PROTEIN CONJUGATES IN GUINEA PIGS

ɴ Katsutani, T Aoki, H Shionoya and F Sagami. *Department of Drug Safety Research, Eisai Co., Ltd., Gifu, Japan.* Sponsor: G K Hanasono

Since anaphylactic reactions are known to be inhibited by the existence of a hapten, it is of interest to examine the inhibitory potential of a drug on anaphylactic reactions in animal models, in relation to prediction of incidence of anaphylactic reactions in clinical use. Cutaneous anaphylactic reactions were provoked by intravenous administration of the corresponding antibiotic-ovalbumin (OVA) conjugate in guinea pigs passively sensitized with antiserum to PCG or LMOX. Inhibition reactions were assessed by co-administration of one of the drugs at doses of 1, 10 and 100 mg/kg with the respective antibiotic-OVA conjugate. LMOX inhibited the reactions from the lowest dose tested, whereas the reactions were not affected by administration of PCG at the highest dose. These results coincide with the previously reported in vitro inhibitory effects of these antibiotics on ELISA for antibiotic-specific antibody analysis[1], and suggest a possible mechanism for the higher incidence of clinical anaphylactic reactions caused by PCG than by a new-generation cephalosporin, LMOX.

1) Katsutani, N. and Shionoya, H. (1993). Int. Arch. Allergy Immunol. 100, 128-134.

325 THE EFFECTS OF TWO MECHANISTICALLY DISTINCT COMPOUNDS ON THE CYTOKINE PRODUCTION BY AN *IN VITRO* T CELL SYSTEM

B P Lawrence, M Meyer, E A Dearstyne, D J Reed, and N I Kerkvliet. *Depts. of Agricultural Chemistry, Biochemistry & Biophysics, and Environmental Health Sciences Center, Oregon State University, Corvallis, OR*

The immune system is a potential target for the toxic effects of many environmental and pharmacologic agents. Moreover, the alteration of growth factors and cytokines has been shown to be the target of chemical-induced toxicity. We used antigen-specific helper T (T_H) clones as an *in vitro* model system study the effects of two mechanistically distinct chemicals on cytokine production. 1,2-Dichloroethane (DCE) is widely used as an industrial solvent and insecticidal fumigant. 17β-Estradiol (17β-Es) is a hormone and is also the prototype for a class of estrogen-like chemicals found in the environment. We have examined the effects of DCE and 17β-Es exposure on cytokine production by both T_H1-type and T_H2-type murine cell lines. Cells were activated with antigen and irradiated syngeneic antigen presenting cells in the absence or presence of DCE or 17β-Es, and the release of cytokines into culture supernatants was measured over a 72 h timecourse. DCE (0.1 to 1.0 mM) caused a dose-dependent increase in the production of interferon gamma (IFNγ) by the T_H1 cell line F1.A.2, and 50% increase in both interleukin (IL)-4 and IL-6 by the T_H2 cell line 10.5.17. These effects were also observed when the polyclonal T cell activator anti-CD3 was used to activate T_H clones. Preliminary studies have shown that 17β-Es (10 nM) treatment decreased IFNγ production by F1.A.2 cells, but not by another T_H1 cell line. 17β-Es did not alter IL-6 production but did suppress IL-4 production by two different T_H2 cell lines (F4 and 10.5.17). The results from these studies suggest that both DCE and 17β-Es are capable of modulating T cell cytokine production.

Supported by NIH Grant ES00040 (NIK and DJR).

326 THE EFFECTS OF DIETHYLSTILBESTROL (DES) IN THE BROWN NORWAY RAT MODEL OF AUTOIMMUNE DISEASE

N. Harper, L. F. Butterworth, D. W. David, and K. L. White Jr. *Dept. Pharmacol./Toxicol. Medical College of Virginia, Virginia Commonwealth Univ., Richmond, VA*

The Brown Norway (BN) rat develops an autoimmune disease that is similar to human systemic lupus erythematosis (SLE) but will do so only after chemical exposure to immunomodulatory agents such as mercuric chloride ($HgCl_2$). Like humans with SLE, the BN rat is predisposed to respond immunologically with TH2 response that is directed towards antibody production rather than cell-mediated immune functions; this may be the mechanism by which chemicals induce autoimmune disease in this model. Since estrogen induces a polyclonal activation of B-cells and is implicated in the development of some autoimmune disorders, it was hypothesized that chemicals with estrogenic activity will exacerbate autoimmune disease in the BN rat. DES, a drug with potent estrogenic activity, was used to test the hypothesis. Initial time course studies showed that female Brown Norway rats gavaged with DES (50 mg/kg/day) had increased serum titers of IgE which peaked at week 4. A dose-response study showed that IgE and anti-DNP IgG titers increased in a dose-dependent manner and peaked at a dose of 50 μg/kg/day. Increases in anti-dsDNA IgG and anti-laminin IgG were also observed. The percentage of spenocytes expressing of MHC II were increased and the IL-2 receptor expression was decreased at all doses. The results show that DES increases serum immunoglobulin titers which may exacerbate the onset of autoimmune disease in this model.

327 CELLULAR IMMUNE MEDIATION OF HALOTHANE INDUCED HEPATOTOXICITY

S M Furst and A J Gandolfi. *Department of Anesthesiology, University of Arizona, Tucson, AZ*

Halothane, once the anesthetic of choice for general anesthesia, has declined in use since its association with liver complications with a high potential for lethality. There is ample evidence suggesting halothane hepatitis is an idiosyncratic autoimmune reaction brought about by the formation of neoantigens that have been generated by covalent binding of a halothane biotransformation intermediate. The guinea pig is being developed as a sensitive animal model to investigate an immune-mediated mechanism for halothane hepatitis. Male Hartley guinea pigs were exposed to 1.0% halothane, 40% oxygen for 4 hr. Kupffer cells and lymphocytes were isolated on various days post-exposure. Lymphocyte transformation tests (LTT) using various TFA-antigens as well as Kupffer cells were performed. Using gel electrophoresis and Western blotting techniques, it has been demonstrated that Kupffer cells obtained from halothane treated guinea pigs do carry TFA-adducts as recognized by an anti-TFA antibody. Bands were most prominent by day 2 with the majority diminishing by day seven. No TFA-adducts were detected in any other organ macrophages isolated indicating the immune response to be initiated within the liver by Kupffer cells. The LTT showed a four-fold increase in splenocyte proliferation in response to TFA-guinea pig albumin (GSA). Peak levels of sensitization were observed in splenocytes isolated from guinea pigs 28 days after the animals were exposed to halothane. Stimulation occurred after second and third exposures as well. No significant increase in proliferation could be detected with TFA-lysine or GSA. A ten-fold increase in splenocyte proliferation also occurred in response to Kupffer cells from halothane exposed animals. Autologous splenocytes demonstrated more of a response from treated vs control animals and this could be blocked using anti-MHC II antibody indicating the involvement of MHC II. These results demonstrate Kupffer cells to be the initial cells that present altered proteins in the liver to cells of the immune system. This study provides good evidence that halothane hepatitis is mediated by a cellular immune response.

(Sponsored by Procter & Gamble Pharmaceuticals).

328 IMMUNOTOXICITY OF ULTRAVIOLET (UV) B IRRADIATION: CORRELATION WITH INTERLEUKIN 6 (IL-6) PRODUCTION

M B Lappin[1], R J Dearman[2], I Kimber[2] and M Norval[1]. [1]*Department of Medical Microbiology, University of Edinburgh, Scotland;* [2]*Zeneca Central Toxicology Laboratory, Macclesfield, England*

Susceptibility to UVB-mediated immunotoxicity is known to differ among strains of mice. In the present study we have examined the association between susceptibility to UVB irradiation-induced inhibition of contact sensitization and the production of IL-6 following topical exposure to a contact

allergen. Two strains of mice, BALB/c and C3H/HeN, have been compared that display, respectively, low and medium/high sensitivity to the immunosuppressive effects of UVB light. Topical exposure of BALB/c and C3H/HeN mice to a skin sensitizing concentration (1%) of the contact allergen oxazolone induced vigorous proliferative responses by draining lymph node cells (LNC) of comparable magnitude. Under these same conditions of exposure very substantial differences in the production by draining LNC of IL-6 (measured by enzyme-linked immunosorbent assay) were observed. Whereas supernatants prepared from cultures of BALB/c LNC contained approximately 10ng/ml IL-6, those from C3H/HeN LNC contained on average 0.3ng/ml. Such differences do not, however, reflect a generalized strain variation with respect to IL-6 expression as in each case treatment with oxazolone induced similar increases in the production of this cytokine in the skin. The data reveal an interesting association between susceptibility to UVB-mediated immunosuppression and the ability of activated LNC to elaborate IL-6.

329 α-NAPHTHYLISOTHIOCYANATE STIMULATES NEUTROPHILS TO RELEASE PROTEASES THAT ARE TOXIC TO HEPATOCYTES *IN VITRO*

D A Hill and R A Roth. *Dept. of Pharm./ Tox., Inst. for Environ. Tox., Michigan State University, East Lansing, MI*

Administration of α-naphthylisothiocyanate (ANIT) to rats causes neutrophil-dependent necrosis of bile duct epithelial and periportal parenchymal cells. Previous studies demonstrated that ANIT can activate neutrophils. We hypothesized that proteolytic enzymes from ANIT-treated neutrophils would damage hepatocytes (HCs) *in vitro*. To test this hypothesis, neutrophils were isolated from Sprague Dawley rats and incubated with ANIT for 18 hours. ANIT (6–50 uM) was not toxic to neutrophils as indicated by lactate dehydrogenase release into culture medium. The conditioned medium from ANIT-treated neutrophils (ANCM) was collected, centrifuged, added to isolated HCs and incubated for 8, 16 or 24 hours. Toxicity of HC cultures was indicated by alanine aminotransferase (ALT) release. Conditioned medium collected from neutrophils exposed to 50 or 25 uM ANIT for 16 to 24 hours caused a pronounced release of ALT from HC cultures. The addition of alpha-1-antitrypsin or trypsin/chymotrypsin inhibitor to ANCM-treated HC cultures afforded protection from toxicity. These data suggest that ANIT stimulates neutrophils to release proteases which cause hepatocellular damage *in vitro*. (Supported by NIH grant ES04139).

330 THE EFFECT OF BE INHALATION ON HUMORAL AND CELL-MEDIATED IMMUNITY IN THE C3H MOUSE

J M Benson[1], E B Barr[1], D E Bice[1], K J Nikula[1], S. M. Clarke[2], S. M. Thurlow[2], and D. E. Hilmas[2]. [1]*Inhalation Toxicology Research Institute, Albuquerque NM;* [2]*DynCorp of Colorado, Inc. Golden, CO*

The purpose of this study was to determine the effect of inhaled Be on humoral and cell-mediated Be-specific immunity in the C3H mouse. Groups of female C3H mice were exposed pernasally to Be metal aerosols under conditions resulting in initial lung burdens of 10, 20 and 40 micrograms Be/lung. Before sacrifice, mice were challenged by intradermal injection of $BeSO_4$ in the footpad to evaluate Be-induced delayed-type hypersensitivity. Mice were sacrificed 7, 60, 120 and 240 days later for evaluation of histopathological changes in lung, lung lymphocyte proliferation in response to Be challenge in vitro, and for quantitation of concentrations of Be-specific IgG in serum. Be inhalation resulted in chronic granulomatous pneumonia with multiple discrete interstitial aggregates of lymphocytes. Bronchiole-associated and perivascular lymphocytes were also increased. The severity of the lesions increased with time and Be lung burden. Immunohistochemical staining of lungs from the high dose mice at 240 days post exposure indicated that the majority of the lymphocytes in the aggregates were B cells. Intradermal challenge of mice with Be did not increase footpad thickness at any time post exposure. Isolated lung lymphocytes proliferated in response to the mitogen, phytohemagglutinin, but not in response to Be. Be-specific IgG was detected in 1/7 high-dose mice by 7 days post exposure and 1/6 medium dose and 4/6 high dose mice at 240 days post exposure. Results suggest that Be inhalation affects humoral rather than cell-mediated immunity in the C3H mouse. [Research supported by the Office of Health and Environmental Research, U.S. Department of Energy, under Contract No. DE-AC04-76EV01013.]

331 SYSTEMIC PRIMING ENHANCES PULMONARY IMMUNITY AGAINST RICIN IN MICE

D M Walters, P V Lemley, and D A Creasia. *United States Army Medical Research Institute of Infectious Diseases, Frederick, MD*

Although vaccines have been developed that protect from lethality induced by ricin, we have thus far been unable to induce mucosal immunity against ricin to protect the respiratory tract. In order to better understand the humoral response to ricin vaccine, both systemically and in the respiratory tract, we immunized mice intra muscularly (im), intratracheally (itr), or both 2x with ricin A-chain, challenged them 2 weeks later with a lethal ricin aerosol ($\sim LC_{90}$), and compared anti-ricin IgG and IgA in serum and bronchoalveolar lavage fluid (BALF) before and after ricin aerosol exposure. Mice that were systemically immunized, but not exposed to a ricin aerosol, had 1:10,000 IgG titers, but no detectable IgA in the serum, and had 1:10 IgG titers, and no detectable IgA in BALF. On the other hand, mice that were systemically immunized and exposed to ricin aerosol, had 1:100 IgG titers and 1:100 IgA titers in the serum. BALF from this group of mice contained IgG and IgA titers of 1:100. Mice that were immunized itr had IgG titers of 1:1000, but no IgA in the serum, and had IgG and IgA titers of 1:100 in BALF. Mice immunized only itr did not survive ricin aerosol exposure. Thus, the data presented here suggest that in order to induce pulmonary immunity against ricin, animals must first be systemically immunized and boosted via the respiratory tract.

332 INDUCTION OF PULMONARY ANTI-RICIN IgA BY A RICIN-CHOLERA TOXIN B-CHAIN CONJUGATE

D A Creasia, D M Walters, and H B Hines. *United States Army Medical Research Institute of Infectious Diseases, Frederick, MD*

Some toxins, such as have a direct necrotizing effect when in contact with respiratory mucosal tissue. Although a humoral immune response (IgG) can, and has been, elicited in response to systemic administration of ricin, the humoral response induces a ricin-specific circulating IgG in the serum, but no respiratory IgA is induced to protect the respiratory mucosa. To address this problem, we coupled the known mucosal immunogen, cholera toxin (CT) B subunit, to ricin by a modification of the methods published for covalently linking N-2 fluorenyl succinamic acid to carrier protein. The resulting ricin-cholera mucosal immunogen was purified to a single HPLC peak and was used to immunize mice both systemically and via the respiratory tract for comparison. Both subcutaneously administered ricin-CT conjugate and ricin toxoid (25 μg/mouse, 3x, biweekly) induced anti-ricin IgG in both serum (≥1: 10,000) and bronchoalveolar lavage fluid (BALF) (≥1: 10,000), but no IgA in either serum or BALF. Ricin-CT conjugate and ricin toxoid administered via the respiratory tract (intratracheal instillation) produced anti-ricin specific IgA and IgG response in BALF (≥1: 1000, and IgG titer (≥1: 1000), but no IgA titer in serum. When each group was challenged with a lethal ricin aerosol (~18μg/L), only the systemically immunized mice survived.

333 NITRIC OXIDE PRODUCTION INCREASES IN THE ABSENCE OF ENDOGENOUS GLUCOCORTICOIDS

P. Preziosi and P. Navarra. *Institute of Pharmacology, Catholic University Medical School, L.go F. Vito 1 – 00168 Rome, Italy*

The subacute toxic effects of the anticancer drug hydroxyurea (HU) are markedly increased in adrenalectomized (ADX) rats (80–100% lethality after 5 days of oral treatment with 300–800 mg/kg/day), but these animals can be fully protected against these effects by administration of exogenous glucocorticoids (GCs) (*e.g.* dexamethasone 100 gmg/kg/day). Considering the multiplicity of effects exerted by GCs, this protection might be attributed to several mechanisms.

In studies on enhanced HU toxicity in ADX rats, we found that whole-body nitric oxide (NO) is markedly increased after ADX. Plasma levels of L-citrulline, which is co-produced with NO in equimolar amounts as a result of NO-synthase breakdown of L-arginine, were used as an index of circulating NO concentrations in intact (I) and ADX rats. The following results emerged: Mean plasma L-citrulline pm SEM (expressed in pM) ($n = 5$) after treatment with vehicle (V) was 11.24 pm 0.66 in I rats and 45,900 pm 100 in ADX rats; after HU 10 mg/kg: 12.33 pm 0.32 in I rats and 47,900 pm 420 in ADX rats (the latter, $p < 0.01$ *vs* V treatment); after HU 100 mg/kg: 30.44 pm 1.5 in I rats and 60,770 pm 670 in ADX rats (both, $p < 0.01$ *vs* V treatment); after HU 800 mg/kg: 178 pm 1.1 in I rats and 90,480 pm 500 in ADX rats (both, $p < 0.01$ *vs* V treatment).

Glucocorticoid protection against HU toxicity in ADX rats may depend

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TH2 CYTOKINE mRNA EXPRESSION IN PRIMARY CUTANEOUS CD30-POSITIVE LYMPHOPROLIFERATIVE DISORDERS: SUCCESSFUL TREATMENT WITH RECOMBINANT INTERFERON-γ. Hiroaki Yagi, Yoshiki Tokura, Fukumi Furukawa, Masahiro Takigawa, Department of Dermatology, Hamamatsu University School of Medicine, Hamamatsu, Japan

The administration of recombinant interferon-γ (rIFN-γ) has been approved for the treatment of patients with mycosis fungoides in Japan. We examined therapeutic efficacy of rIFN-γ and alterations of cytokine profile of skin-infiltrating cells in primary cutaneous CD30-positive lymphoproliferative disorders. rIFN-γ was injected intravenously or locally in two cases of Ki-1-positive lymphoma (KiL) and two cases of lymphomatoid papulosis type A (LyP). In all cases, except for one KiL, the skin lesions were improved and the numbers of skin-infiltrating CD30-positive cells were remarkably decreased after the injection of rIFN-γ. RT-PCR was performed for detecting cytokine mRNA of the dermal infiltrating cells in three KiL and three LyP. mRNA for IL-4 and IL-10 was detected in all six cases, whereas mRNA for IL-2 and that for IFN-γ were transcribed in two and one cases, respectively. Compared with normal controls from ten healthy donors, the cytokine profile of dermal infiltrating cells in these cases was revealed to show Th2-type skewing. In four cases treated with rIFN-γ, mRNA expression was compared before and after the administration of rIFN-γ. In three cases with a good therapeutic response to rIFN-γ, the transcription of mRNA for IL-4 and IL-10 was downmodulated by the injection of rIFN-γ. These findings suggest that the skin-infiltrating cells in primary cutaneous CD30-positive lymphoproliferative disorders have a cytokine profile of Th2 and that the administration of rIFN-γ improves the disease condition by inhibiting the growth of Th2 tumor cells.

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TREATMENT OF MITOGEN IN THE MURINE LOCAL LYMPH NODE ASSAY : ABILITY TO DISCRIMINATE BETWEEN ALLERGENS AND IRRITANTS. Jong-Ho Park, Jae-Sook Koh, Kyung-Mee Yang, Won-Jae Park, Safety & Analysis Research Center, Pacific Corporation, Kyounggi-do, Korea.

The murine local lymph node assay has been generally used as a method of identifying the contact allergens, in which sensitizing activity was measured as a function of induced proliferative responses in lymph nodes draining the site of application. But according to recent studies, non-sensitizing irritants, such as SDS, also caused lymphocyte proliferation. In this study, tested irritants, i.e. sodium dodecyl sulfates(SDS), triton X-100, also induce lymphocyte proliferation, which was not distinguishable from the results with allergens. To develop the method of discrimination between allergens and irritants, we used mitogens concanavalin A and phytohemagglutinin A(PHA) of various concentrations, and the results were compared to non-treated lymphocyte proliferation. As a result, we observed that the mitogens at very low concentrations, below 0.5 μg/ml, remarkably stimulated lymphocytes in allergen group ; 0.5 μg/ml of mitogen induced more than tenfold greater proliferation rate than that of the non-treated group. But in vehicle control and irritant group, the proliferation rate was less than fivefold at the same concentration of the mitogens. These results show that treatment with mitogens of low concentrations is a useful method of discrimination between allergens and irritants.

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THE EFFECT OF *IN VIVO* ULTRAVIOLET-B EXPOSURE ON ACCESSORY FUNCTION AND THE PHENOTYPE OF LYMPH NODE DENDRITIC CELLS DURING THE INDUCTION PHASE OF CONTACT SENSITIZATION. Mary Norval, Michael B. Lappin, Ian Kimber[1], Department of Medical Microbiology, University of Edinburgh Medical School, Edinburgh, Scotland. [1]Zeneca, Central Toxicology Laboratory, Macclesfield, UK.

Exposure to low doses of ultraviolet-B (UVB) irradiation before sensitization results in suppressed contact hypersensitivity responses. *In vitro* investigations have suggested that one influence of UVB light is to modify the function of Langerhans cells (LC) as antigen presenting cells. To investigate further the effect of UVB on LC activity, changes in LC-derived lymph node dendritic cells (DC) were studied following exposure of C3H/HeN mice to an immunosuppressive dose of UVB (1440J/m^2) 48 and 24 hrs prior to skin sensitization with fluorescein isothiocyanate (FITC) or oxazolone. In functional studies the ability of DC prepared from the draining lymph nodes of contact sensitized mice were examined for their ability to induce hapten-specific secondary T lymphocyte proliferative responses or mixed lymphocyte responses. In neither case was the activity of DC affected by local exposure to an immunosuppressive dose of UVB. The migration of LC from the epidermis to the draining lymph node in response to contact sensitization is associated with a rapid increase in the expression of several membrane determinants necessary for effective antigen presentation, including intercellular adhesion molecule-1 (ICAM-1; CD54), B7-2 (CD86) and Ia antigen. The expression of these molecules was identical on DC isolated from UVB irradiated and from control unirradiated mice. It is concluded that the immunosuppressive effect of UVB on the cutaneous immune system may not necessarily reflect changes in LC phenotype or function.

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IMMUNOPROTECTION AGAINST A MURINE CD4$^+$ T CELL LYMPHOMA IS MEDIATED BY TUMOR-SPECIFIC CYTOTOXIC T CELLS. C. Berger, S. Yang, A. Felli, M. Perez & R. Edelson, Depart of Dermatology, Yale Univ. & Columbia Univ., New Haven, CT & NY, NY.

The 2B4.11 hybridoma is a fusion product of the BW5147 thymoma and pigeon cytochrome c primed T lymphoblasts, and serves as a murine model of T cell lymphoma. We have previously shown that immunoprotection against lethal tumor challenge occurs after immunization of mice with 8-methoxypsoralen and ultraviolet A (8-MOP UVA) treated tumor cells. This immunoprotection is now shown to be mediated by induction of CD8$^+$ cytotoxic T cells that specifically lyse the tumor cells.

Tumor cells (5-10x10^6 cells) were treated with 200 ng/8 MOP and 1 J/cm^2 UV A and injected 2x/wk for 4 wk. Mice were challenged with 5x10^6 viable tumor cells. Immune mice were sacrificed and the splenocytes phenotyped and cultured with γ-irradiated tumor and IL2. Cytotoxicity was determined in a ^{51}chromium release assay.

Control mice (N=10) died within 4 wk. Two of 15 treated mice died (13%) at 5 wk, and the remainder survived without disease. Splenocytes from treated mice were 85% CD8$^+$ and were cytotoxic for 2B4.11 tumor targets (40-66% lysis). Cytolysis of the BW5417, C10.9 hybridoma and natural killer cell RMA-S targets was 13%.

The results confirm that immunoprotection to lethal tumor challenge is mediated by tumor-specific cytolytic CD8 cells induced after immunization with 8 MOP-UV A treated tumor cells. The murine model predicts that similar tumor immunity may be induced in cutaneous T cell lymphoma patients treated by photopheresis. CD8$^+$ anti-tumor T cells may be used to isolate and characterize class I associated tumor peptides.

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CUTANEOUS T CELL LYMPHOMA LYMPHOCYTES EXPRESS CELL SURFACE HEAT SHOCK PROTEIN Zuming Dong, Richard Edelson, Emil Bisaccia, and Carole Berger, Department of Dermatology, Yale Univ. and Morristown Memorial Hospital, New Haven CT and Morristown, NJ.

BE2, a monoclonal antibody, recognizes a monomeric 78 kd cell surface protein on lymphocytes from patients with cutaneous T cell lymphoma (CTCL), adult T cell leukemia, Epstein-Barr virus (EBV-B) transformed B cell lines, and activated normal lymphocytes. The ubiquitous expression of the BE2 molecule on the cell surface of CTCL tumor cells allowed its use as a diagnostic and prognostic reagent and suggested that it played an important role in the immunobiology of the malignancy.

The BE2 protein was isolated and purified by 1 and 2 dimensional electrophoresis and sequenced. The sequence was highly homologous to the 78 kd heat shock protein (HSP), BIP, an endoplasmic reticulum (ER) chaperonin. The relationship between BIP and BE2 was supported by the demonstration that BE2 strongly binds to ATP. However, polyclonal and monoclonal reagents that recognize 70 and 78 kd HSP do not detect the BE2 antigen on the surface of CTCL or EBV-B lymphocytes.

BE2 may represent a novel member of the HSP family that is modified for cell surface expression and may serve as a cytoplasm to cell surface chaperonin for peptides and proteins including empty class I MHC heavy chain and T cell receptor molecules. These findings may provide the basis for definition of a new route for exogenous and endogenous peptide transport, between the cell surface and the ER.

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IMMUNOCYTES TRIGGER INDUCTION OF FULLY EVOLVED PSORIATIC PLAQUES (PP SKIN) USING SCID MICE : HUMAN SKIN XENOGRAFTS. B.J. Nickoloff, T. Wrone-Smith, Departments of Pathology and Dermatology, University of Michigan, Ann Arbor, MI.

Recent studies have implicated a key role for various immunocytes including T cells and antigen-presenting cells (APCs) in the pathogenesis of psoriasis. However, due to the lack of an appropriate animal model it has not been possible to definitively establish that the psoriatic phenotype can be transferred by specific immunocytes. To fill this experimental void, keratome samples (including dermis and epidermis) of symptomless (PN) skin of psoriatic patients (N=4) were transplanted onto SCID mice. 3-4 weeks following engraftment, 200 μl containing various stimuli were injected intradermally either once or twice, and the grafts harvested 2-4 weeks later. Stimuli included: PBS (control); GM-CSF (400 μg); IFN-γ (1 μg); superantigens (SA; SEB and SEC2); LPS (1 μg); and autologous immunocytes (1-2 x 10^6 cells) including either: PP skin-derived dermal dendritic (DD) cells and T cells; or Ficoll-Hypaque derived blood mononuclear cells (FH cells) cultured with or without SA and IL-2. While some increase in epidermal thickness occurred with the soluble mediators, full-fledged psoriatic plaques were evident only with the immunocyte-containing cellular injections. Maximal epidermal thickness measurements (microns) are: PP skin pre-transplant = 513 ± 97; PN skin pre-transplants = 121 ± 14; PN skin post-transplant after injection with; PBS = 125 ± 16; GM-CSF = 174 ± 12; IFN-γ = 176 ± 16; SA = 216 ± 32; LPS = 218 ± 25; DD = 289 ± 22; FH-unactivated = 359 ± 35; FH-activated = 522 ± 63. Microscopic and immunohistochemical analysis of PN skin injected with FH cells revealed unequivocal hallmarks of PP skin including thick parakeratotic scale, prominent elongation of rete pegs, loss of granular cell layer, suprabasilar mitotic figures, and infiltration of human T cells (predominantly CD8) in epidermis with dermal accumulation of APCs as well as CD4 positive T cells. Lesional keratinocytes were diffusely positive for proliferating cell nuclear antigen, as well as being ICAM-1 and HLA-DR positive.

In summary, this is the first demonstration using an animal model that complete phenotypic conversion of PN skin to PP skin can be accomplished. Moreover, the plaques were directly caused by injection of immunocytes into the dermis, which re-distributed themselves in a non-random fashion to recapitulate entirely the appropriate microscopic appearance of idiopathic psoriatic lesions.

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UVB IRRADIATION-ENHANCED IL-6 PRODUCTION AND mRNA EXPRESSION IS MEDIATED BY IL-1α IN CULTURED HUMAN KERATINOCYTES. Jin Ho Chung, Sang Ho Youn, Woo Seok Koh, Hee Chul Eun, Kwang Hyun Cho, Kyung Chan Park and Jai Il Youn, Department of Dermatology, Seoul National University College of Medicine, Seoul, Korea.

Ultraviolet B radiation may trigger cutaneous inflammatory responses by directly inducing epidermal keratinocytes to elaborate specific cytokines, such as IL-1 and IL-6. Because IL-1 is a potent inducer of IL-6, one may speculate that the release of IL-6 by keratinocytes following UV exposure is mediated via the release of IL-1 in an autocrine or paracrine manner. The purpose of this study was to evaluate the effects of UVB on IL-1α and IL-6 mRNA expression by keratinocytes, and to investigate whether this UVB irradiation-enhanced IL-6 was mediated by IL-1α.

We demonstrated that UVB irradiation upregulated the IL-1α mRNA at a lower dose (15 mJ/cm^2) and then downregulated IL-1α mRNA expression at high doses (30 - 40 mJ/cm^2). The kinetic profile of IL-1α mRNA expression showed a biphasic response, the early increase by 1 h after UV and the secondary increase at 6 h after UV. On the other hand, the expression of IL-6 mRNA was increased with increasing doses of UVB (0 - 45 mJ/cm^2), and showed a single peak at 6 h post-UV. These results may indicate that UVB radiation could regulate the expression of IL-1α and IL-6 mRNA in keratinocytes by different mechanisms.

We have demonstrated that anti-human IL-1α antibody inhibited the UV-induced IL-6 production and mRNA expression in cultured keratinocyte. The addition of recombinant IL-1α to the medium increased the IL-6 synthesis, and augmented IL-6 production and mRNA expression in cultured human keratinocytes by UVB irradiation. These results support the hypothesis that UVB irradiation-enhanced IL-6 production and mRNA expression may be mediated by IL-1α.

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MATERNAL AUTOIMMUNE RESPONSE TO RECOMBINANT RO/SSA AND LA/SSB PROTEINS IN JAPANESE NEONATAL LUPUS ERYTHEMATOSUS. S. Miyagawa*, T. Fukumoto*, K. Hashimoto[‡], T. Hachiya[†], A. Yoshioka[‡], and T. Shirai* Departments of *Dermatology and [‡] Pediatrics, Nara Medical University, Nara; and [†] InaLaboratory, Medical & Biological Laboratories Co, Nagoya, Japan

Background: Neonatal lupus erythematosus (NLE) is a syndrome characterized by dermatitis and congenital heart block (CHB). The disease is mostly associated with transplacental passage of maternal anti-Ro/SSA and/or La/SSB antibodies.

Objective: To examine maternal autoimmune response to recombinant Ro/SSAand La/SSB proteins in NLE with a single ethnic background.

Methods: This study examined 12 NLE infants and their mothers. Serum samples were tested by enzyme-linked immunosorbent assay for reactivity withfull-length recombinant human 60-kd Ro/SSA, 52-kd Ro/SSA, and 48-kd La/SSB proteins.

Results: All 10 infants with skin lesions and/or CHB had maternal antibodies reactive to both the 60- and 52-kd Ro/SSA polypeptide components of the Ro/SSA particle. Anti-60-kd Ro/SSA without anti-52-kd Ro/SSA, or *vice versa*, was found in mothers of infants who had only hepatic manifestation of NLE. Nine of 12 NLE infants had anti-48-kd La/SSB.

Conclusion: In the majority of Japanese infants with NLE, maternal anti-Ro/SSA autoimmune response was directed against both of 60-kd and 52-kd recombinant Ro/SSA proteins. There was no profile of anti-Ro/SSA and La/SSB response unique to mothers of children with CHB or cutaneous manifestations of NLE.

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INDUCTION OF TNFα EXPRESSION BY UV, DNA DAMAGE AND UROCANIC ACID. Adrienne O'Connor, Jeannie Kibitel, Jon Klein, Bruce Beutler* and Daniel Yarosh. Applied Genetics Inc., Freeport, New York and *Howard Hughes Medical Institute, UT Southwest Medical Center, Dallas, Texas

Keratinocytes elaborate an array of cytokines after exposure to UV, including TNFα. We tested the hypothesis that modulation of DNA damage and repair would alter the expression of this cytokine. The cell line XP12BE (SV40-transformed fibroblasts from xeroderma pigmentosum complementation group A) was chosen for study in order to eliminate endogenous DNA repair. Several clones were selected after co-transfection with one plasmid carrying the TNFα promoter linked to the CAT gene, and a second plasmid containing the neo gene for selection for resistance to G418. Both UV-B and UV-C induced CAT at doses lower than those reported for repair-proficient cells. Increasing repair of cyclobutane pyrimidine dimers by treatment of UV-B irradiated cells with T4N5 liposomes encapsulating the DNA repair enzyme T4 endonuclease V reduced expression of CAT. On the other hand, production of double-stranded breaks by treatment with Hind III restriction enzyme encapsulated in liposomes produced only a modest increase in CAT. Liposomes containing heat-inactivated enzymes did not produce these responses. No difference was found in CAT expression between cells incubated with 0.04% trans-urocanic acid and those incubated with 0.04% UV-B-irradiated urocanic acid (50% cis- and 50% trans- isomer). These results suggest that DNA damage, such as UV-induced photoproducts (e.g. cyclobutane pyrimidine dimers) and double stranded breaks, can induce TNFα expression.

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INTERLEUKIN 12 (IL-12) BREAKS UV-INDUCED TOLERANCE. Agatha Schwarz, Stephan Grabbe, Yoshinori Aragane, Kirsten Sandkuhl, Helge Riemann, Thomas Luger, Marek Kubin*, Giorgio Trinchieri*, and Thomas Schwarz, Ludwig Boltzmann Institute for Cellbiology and Immunobiology, Department of Dermatology, University Münster, Münster, Germany, *The Wistar Institute, Philadelphia PA

We recently showed that intraperitoneal (i.p.) injection of IL-12 prevents UVB-induced local suppression of contact hypersensitivity (CHS). Since application of haptens on UVB-exposed skin does not only result in the failure to induce CHS but also induces hapten specific tolerance, we studied whether IL-12 can break UV-induced tolerance. C3H/HeN mice were sensitized with dinitrofluorobenzene (DNFB) through skin exposed to low dose UVB on 4 d and ear challenge performed 5 d later. UV-treated mice resensitized 14 d after the first challenge through non-UV-exposed skin displayed hapten-specific tolerance, whereas UV-exposed mice injected i.p. with IL-12 before *re*sensitization produced a significant ear swelling response suggesting that IL-12 can *break* UV-induced tolerance. Whereas adoptive transfer of spleen cells from UV-irradiated mice inhibited sensitization of the recipient mice, no inhibitory effect was observed after transfer of spleen cells from UV-exposed and IL-12 treated mice. Depletion of either $CD4^+$ or $CD8^+$ T-cells revealed that UV-induced suppression is transferred via $CD8^+$ cells. To determine whether IL-12 overcomes tolerance by inhibiting $CD8^+$ suppressor cells or by activating $CD4^+$ effector cells, splenocytes from UV-exposed, DNFB treated and IL-12 injected mice were depleted from $CD4^+$ cells and transferred into naive mice which were subsequently sensitized. Transfer of CD4 depleted splenocytes from UV-irradiated and IL-12 treated mice still resulted in lack of suppression of sensitization in recipient animals suggesting that IL-12 rather acts on $CD8^+$ suppressor cells than on $CD4^+$ effector cells.

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RECOVERY FROM THE IMPAIRED TH1 RESPONSES IN ATOPIC DERMATITIS PATIENTS BY IL-12 OR ANTI IL-4, ANTI IL-10 ANTIBODIES. Kazushi Urano, Takashi Matsuyama, Rie Urano, Itsuro Matsuo, Muneo Ohkido, Hideyuki Ozawa*, Sonoko Habu*, and Takashi Nishimura*, Department of Dermatology and *Department of Immunology, Tokai University School of Medicine, Isehara, Japan.

The impaired Th1 immune response and enhanced Th2 response have been considered as one of the causative factors of atopic dermatits(AD). IL-12 has a capability of stimulating Th1 response and inhibits Th2 response. We have already reported that the addition of IL-12 with Dermatophagoises pteronyssinus antigen into the culture of peripheral blood nomonuclear cells(PBMC) from AD patients recovered the IFN-γ production to normal level. In the present study, we ask whether anti IL-4 mAb and anti IL-10 mAb in addition to IL-12 can modulate the impaired Th1 response of AD patients in terms of superantigen induced IFN-γ production of activated T cells. Culture of PBMC from healthy donors with staphylococcal enterotoxin A or anti CD3 antibody caused the high levels production of IFN-γ. In contrast, PBMC from AD patients produced low levels of IFN-γ. However, the simultaneous addition of anti IL-4 and anti IL-10 mAbs into the culture of PBMC from AD patients resulted in the high level of IFN-γ production. PBMC from AD patients spontaneously produced IL-4 but not IFN-γ, indicating IL-4 might be a key inhibitory factor for IFN-γ production. These results suggested that abnormally activated Th2 type cytokines suppress the Th-1 derived IFN-γ production in AD patients and IL-12 may become an effective therapeutic reagent for AD.

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INTERLEUKIN-6 PRODUCTION IN THE SKIN AND LYMPH NODES OF UVB RESISTANT AND SUSCEPTIBLE MICE. Michael B. Lappin, Rebecca J. Dearman[1], Mary Norval, Ian Kimber[1], Department of Medical Microbiology, University of Edinburgh Medical School, Edinburgh, Scotland. [1] Zeneca, Central Toxicology Laboratory, Macclesfield, UK.

Mice of BALB/c and C3H/HeN strains are considered to be resistant and susceptible respectively to suppression of contact hypersensitivity induced by UVB irradiation. We have compared here the ability of draining lymph node cells (LNC) from BALB/c and C3H/HeN strains to elaborate interleukin-6 (IL-6) *in vitro* following topical exposure to the contact sensitizer, oxazolone. Consistent with previous investigations it was found that LNC prepared from sensitized BALB/c mice produced high levels of IL-6 in the culture medium (around 8-10ng/ml); the main or exclusive source of which is believed to be dendritic cells (DC). In contrast, draining LNC from C3H/HeN mice exposed in an identical way to oxazolone produced only low levels of IL-6 (≈0.3ng/ml). Such differences were not attributable to congenital variation between these strains with respect to IL-6 production as comparable levels of spontaneous and inducible cutaneous expression of this cytokine were measured in BALB/c and C3H/HeN mice following, respectively, topical exposure to vehicle alone or oxazolone. In addition when LNC from oxazolone treated C3H/HeN mice were disrupted by sonication, IL-6 was released into the supernatant (≈2.2ng/ml), suggesting a failure in cytokine secretion rather than production. These data indicate that there may exist an association between susceptibility to the immunosuppressive effects of UVB irradiation and the ability of skin draining lymph node cells to synthesize and/or secrete IL-6 following immune activation.